Cellular Prion Protein: a potential biomarker of TBI

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Abstract

Traumatic brain injury (TBI) is considered a silent epidemic affecting millions of people worldwide. Mild TBI (mTBI) is especially a concern as it is the most prevalent form of TBI suffered by individuals who participate in high-contact sports or even the military. Recently, clinicians have emphasized assessing concentration of proteins within biological fluids that can serve as surrogate biomarkers of TBI. Many proteins have been investigated, but none has examined the cellular prion protein (PrP^C), which is expressed abundantly along the extracellular domain on plasma membrane lipid rafts within the central nervous system, and is associated with various important functions. Furthermore, there is mounting evidence that PrP^C release from the CNS may be indicative of neurodegeneration, neurotoxicity, and dysregulation of normal signaling pathways, all of which are staples of TBI. Given PrP^C's extracellular attachment, it is possible that mechanical stretching and shearing forces due to head impact, acceleratory forces to the head, or even oscillating blast exposure can result in PrP^C dislodgement allowing it to collect within the systemic circulation. Moreover, sustained secondary injury mechanisms such as neuroinflammation and oxidative stress for example are known to upregulate PrP^C and even cause its release potentially leading to prolonged elevation of plasma PrP^C concentration following TBI.

Using an advanced blast simulation apparatus, anesthetized adult Sprague-Dawley rats were subjected to head-only exposure to helium gas-driven primary blast wave of varying intensities. Plasma samples were collected 24 hours following blast exposure and assayed for PrP^C analyte concentration as well as being used for comparative immunoblotting against other TBI biomarkers. Furthermore, pre-clinical human plasma samples were collected from athletes following concussion to be analyzed for PrP^C concentration against baseline samples collected during the offseason as well as non-athlete controls. Plasma PrP^C concentration values were

determined by sensitive quantification using a PrP^C-specific enzyme linked immunosorbent assay (ELISA) and found that PrP^C levels are increased following blast exposure, and partly mitigated by protective head covering. Likewise, human samples show that PrP^C plasma concentration shows an increased trend following concussion and are significantly elevated when compared to overall normal values. The results demonstrated within this thesis demonstrate that PrP^C is a potentially novel biomarker for detection of TBI and further alludes towards the protein's involvement in the complex TBI pathology.

Published works

Articles in refereed journals resulting from work presented in this thesis:

Pham N, Dhar A, Khalaj S, Desai K, Taghibiglou C. (2014) Down regulation of brain cellular prion protein in an animal model of insulin resistance: possible implication in increased prevalence of stroke in pre-diabetics/diabetics. Biochem Biophys Res Commun. 448(2): 151-6

Pham N, Sawyer TW, Wang Y, Rastgar Jazii F, Vair C, Taghibiglou C. (2015) Primary Blast-Induced Traumatic Brain Injury in Rats Leads to Increased Prion Protein in Plasma: A Potential Biomarker for Blast-Induced Traumatic Brain Injury. J Neurotrauma. 32(1): 58-65

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DEDICATION

I dedicate this thesis to my loving family.

For your support, your understanding, and your overwhelming patience.

I would not be where I am without your unyielding belief in me.

To my brother for being my confidante and best friend;

To my father who taught me stubbornness when things get tough;

And to my mother who showed me that hard work and a gleaming attitude

can take you far in life.

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List of Abbreviations (alphabetical order)

Aβ	Amyloid β
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
ALS	Amyotrophic lateral sclerosis
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	Beta-secretase 1
BBB	Blood brain barrier
BOP	Blast over pressure
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
˚C	Degrees Celsius
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
CSF	Cerebral spinal fluid
CTE	Chronic traumatic encephalopathy
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
GABA	γ-Aminobutyric acid

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acid protein
GPI	Glycosyl-phosphatidyl-inositol
HAND	HIV-associated neurocognitive disorder
HIV	Human immunodeficiency virus
IR	Immunoreactivity
M	molar
MBP	Myelin basic protein
NFH	Neurofilament heavy chain
NMDA	*N*-Methyl-D-aspartic acid
NSE	Neuron specific enolase
O/N	Overnight
OR	Octapeptide repeat
PD	Parkinson's disease
PBB	BSA in PBS
PBS	Phosphate buffered saline
Prnp	Prion protein gene
PrP^{C}	Cellular prion protein
PrP^{Sc}	Scrapies prion protein
PSEN	Presenilin-1
RBC	Red blood cell
SBDP	Spectrin breakdown product
SCAT3	Sports concussion assessment tool 3

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
STAT1	Signal transducers and activators of transcription 1
S100B	S100 calcium binding protein B
TBI	Traumatic brain injury
bTBI	blast-induced TBI
mTBI	mild TBI
TCPS2	Tri-council policy statement 2
TNFα	Tumor necrosis factor α
TSE	Transmissible spongiform encephalopathy
UCH-L1	Ubiquitin carboxy-terminal hydrolase L1

Introduction

Sport-related concussions and blast-induced traumatic brain injury (b(TBI) are very similar, and in most cases are considered as mild TBI (mTBI). Sports are one of the leading causes of mTBI among children and young adults. While military service members abroad are highly at risk for blast exposure. This especially pertains to recent military conflicts in the Middle East where the use of explosive munitions and improvised devices have been the hallmark of indigenous militant forces. Despite several clinical symptoms and manifestations, it is believed that the majority of sports-related mTBI and bTBI are underreported by the injured and/or overlooked by clinicians. These undiagnosed individuals are at further risk of repeat injury or exposure without proper precautionary measures taken, which can be particularly disastrous and increases the likelihood of developing long-term neurodegeneration. Current standards of care rely on cognitive testing and diagnostic medical imaging techniques, but many injury cases can be undetected due to lack of distinguishing signs. It is thus necessary to have access to more reliable and easy to use quantitative diagnostic measurements to identify those suspected of sustaining TBI. Protein biomarkers in biological fluids provide clinicians a surrogate means to determine brain injury as well as the degree of injury and potential outcome. Various blood plasma proteins have been investigated recently, with some showing promise and others remaining controversial amongst investigators. However, it is important to continue searching for protein biomarkers of TBI not only to provide a quicker, objective, and less invasive method of diagnosis, but also to divulge the complex pathology of TBI, which is still not fully understood. A protein located within the central nervous system (CNS) involved in many important neuronal functions is PrP^{C}, which can potentially be a novel marker of TBI.

The cellular prion protein (PrP^C) is a loosely associated lipid raft protein ubiquitously expressed throughout the body, but is primarily located within the CNS. PrP^C has been linked to several important physiological functions that suggest the protein is involved in normal CNS function as well as being neuroprotective. Given PrP^C's loose attachment via its glycosyl-phosphatidyl-inositol (GPI) anchor along the extracellular surface of the plasma membrane, it is possible that given sufficient mechanical force or shear stress as a result of mild and blast TBI, PrP^C may dislodge off the neuronal lipid rafts, eventually ending up in circulation. Acute TBI primary injury mechanisms such as contusion, axonal shearing, diffuse neuronal injury and cell death, damaged blood vessels leading to hemorrhage and ischemia, and increased CNS permeability due to damaged meninges and blood brain barrier (BBB) can all contribute towards increased neuronal PrP^C levels within the blood. Furthermore, prolonged secondary injury mechanisms following TBI, typically involving biochemical cascades triggered from the primary injury, such as neuroinflammation, oxidative stress, neurotoxicity, apoptosis, as well as central immune response have been shown to increase PrP^C expression or release from the CNS. PrP^C levels in biological fluids such as cerebral spinal fluid (CSF) and plasma have been previously used as a potential biomarker for certain pathological conditions including prion diseases (Picard-Hagen, et al. 2006), Alzheimer's disease (Volkel, et al. 2001), ischemic stroke (Mitsios, et al. 2007), HIV-associated neurodegeneration, and dementia (Meyne, et al. 2009; Roberts, et al. 2010). All of these conditions share some aspect of dysregulated neuronal functioning in common with TBI, which suggests that PrP^C is a potential biomarker for TBI as well. Additionally, recent studies have shown that patients with cerebrovascular disease or vascular endothelial damage had higher levels of plasma PrP^C than control values (Krupinski, et al. 2008; Simak, et al. 2002; Starke, et al. 2002). As the gross pathology of TBI often includes diffuse

microlesions throughout the cerebrovasculature as well as damage to the BBB, there may be additional non-neuronal derived PrP^C released into the system circulation following TBI. In summary, there is a wealth of evidence that suggests that CNS-localized soluble PrP^C is released into the systemic circulation following TBI.

This thesis will discuss the background information and rationale behind exploring the use of PrP^C as a novel TBI biomarker. The design of an animal blast exposure experiment as well as a human pilot study in sports concussion will be described along with the resulting data collected. From these findings, this thesis will show that PrP^C is a novel biomarker of TBI.

Section 1. Cellular Prion Protein (PrP^C)

1.1. Background

Stanley Prusiner first discovered the cause of a rare neurodegenerative disease affecting sheep and goats called scrapies, which resulted in unusual behavior often accompanied by scraping, hence the name, of their fleece on trees and rocks to relieve their itching sensation (Prusiner 1982). Prusiner discovered that the scrapies causing agent was insensitive to nucleic acid modification but was partially sensitive to various protease treatments, thus determining the causative agent to be made of protein, despite its high resistance to digestion. From this finding, Prusiner coined the new term "prion" for small proteinaceous infectious particle. Investigators were able to soon after purify the infectious scrapies prion protein (PrP^{Sc}) and found that besides it being the cause for disease in animals, there was a strong likelihood that a similar protein purified from humans with neurodegenerative Creutzfeldt-Jakob disease (CJD) could also cause disease in humans as both share common structural features, antigenicity, and resistance to proteinase K hydrolysis (Bendheim, et al. 1985; Prusiner 1986).

Using purified preparations of hamster brains infected with scrapies, the cDNA sequence of the infectious PrP^{Sc} was mapped out revealing a single copy gene (*Prnp*), which encodes for a 253-residue protein, on human chromosome 20 present in both infected and non-infected brain extracts, as well as in murine and human brains (Oesch, et al. 1985). Furthermore, this study found that specific antiserum detected prion protein in infected brain tissue, and to a lower extent in non-infected tissue. After proteinase K digestion, however, prion protein in infected neural tissue remained while that in non-infected brains were completely degraded. These findings provided evidence that prion protein was normally expressed in neural tissue, and that the infectious PrP^{Sc} was likely derived from this endogenously expressed protein that somehow

shows higher protein content, later discovered to be due to aggregated plaque formation, as well as becoming resistant to protease in infected tissue, likely through post-translational processing (Aguzzi & Calella, 2009). Efforts to determine the chemical trigger for the synthesis of the infectious PrP^{Sc} from endogenous cellular prion protein (PrP^{C}) found no likely candidates, suggesting pathogenesis is propagated by conformational changes in the normal protein. Fourier-transform infrared spectroscopy analysis determined that although PrP^{C} and PrP^{Sc} possess the same amino acid sequence, their secondary structures were extremely different (Pan, et al. 1993). PrP^{C} is composed of higher α-helical content (45%) with two short stretches of β-sheets (3%) whereas the misfolded PrP^{Sc} conversely has lower α-helices (30%) and much higher β-sheets (45%) (Pan, et al. 1993). This drastic conformational change is responsible for PrP^{Sc}'s resistance to proteolysis as well as promoting misfolding conversion, aggregation, and formation of amyloid fibrils and plaques in neuronal tissue that can eventually cause apoptosis and death of the host (Aguzzi and Calella 2009). Thus, prions are unique in that both the infectious agent and target protein are the same.

Since its discovery, PrP^{C} is often studied for its role as the causative precursor agent for the neurodegenerative diseases of transmissible spongiform encephalopathies (TSEs) in both humans (i.e. Kuru, CJD) and animals (i.e. Scrapies, Mad Cow disease), but research over the past decade has revealed the normal protein to be involved with many physiological processes. The PrP^{C} has been identified in many mammalian species and other vertebrates sharing similar sequence and structural elements with little variation across, suggesting the protein is evolutionarily conserved (Wopfner, et al. 1999). The normal PrP^{C} isoform is constitutively expressed throughout the CNS by neurons, between synapses, astrocytes, axons, brain

microvascular endothelial cells, and microglia (Bremer, et al. 2010; Brown 2001; Brown and Mohn 1999; Fournier, et al. 2000; Moser, et al. 1995; Viegas, et al. 2006). Within the brain, PrP^C is most concentrated within the hippocampal formation; septal, caudate, and thalamic nuclei; dorsal root ganglia cells; and large-diameter dorsal root axons (Bendheim, et al. 1992). PrP^C is a 32 kDa glycoprotein located along the outer leaflet of the plasma membrane in dynamic lipid raft microdomains attached by a glycosyl-phosphatidyl-inositol (GPI) anchor (Stahl, et al. 1992). PrP^C is constitutively recycled between the cell surface and endocytic compartment via clathrin-mediated internalization with the majority of protein (95%) returning to the cell surface intact and the remainder being proteolytically digested (Shyng, et al. 1994; Shyng, et al. 1993). PrP^C expression in extracerebral tissues can be detected in circulating leukocytes, heart, skeletal muscle, lung, intestinal tract, spleen, testis, ovary, and other organs indicating extraneous non-neuronal roles for this protein. However, PrP^C is predominantly expressed within the brain and is involved in important neurological functions.

1.2. Structure

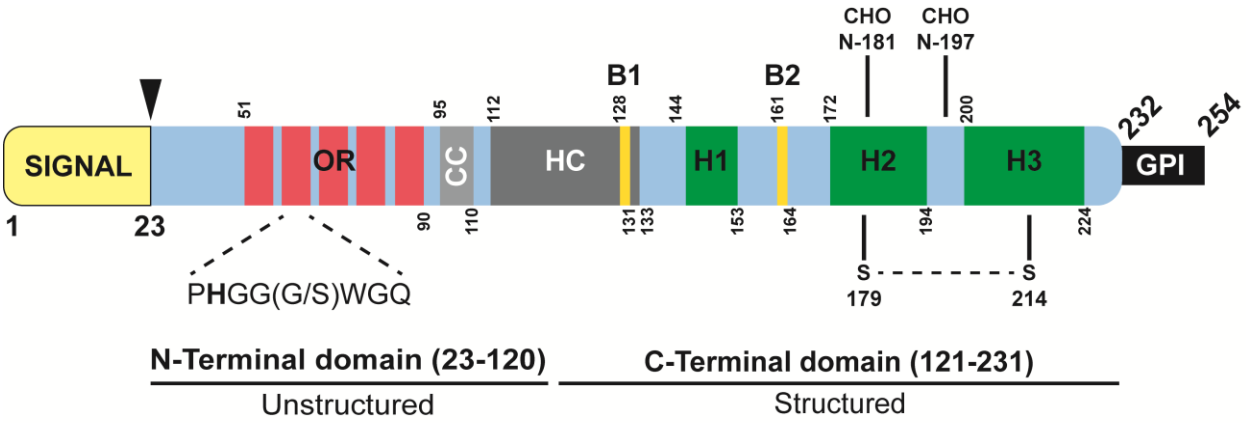
The full-length 253 amino acid long prion precursor polypeptide is synthesized and then translocated into the endoplasmic reticulum (ER) where it undergoes post-translational modifications before reaching its mature form. Within the ER lumen, PrP^C is cleaved at both its amino (N)-terminal signal peptide and hydrophobic carboxy (C)-terminal segment leaving a 208 amino acid long protein in the process. PrP^C undergoes N-linked glycosylation as carbohydrate moieties are added at residues N181 and N197 within the ER that serve the function of stabilizing the protein's structure (Zuegg and Gready 2000). Additionally, a GPI anchor moiety attaches at position 230 within the ER allowing PrP^C to link to the cell's plasma membrane and

is essential for the protein to be cycled back and forth from the endosome. Aside from secreted soluble PrP^{C}, which is predominantly extracellular, there are also two variant forms of PrP^{C} that can span the plasma membrane with either the N- or C-terminus facing outwards from the cell (Stewart, et al. 2001). The mechanism determining the amount of formation for these three variants is not yet understood.

The N-terminal proximal half is largely unstructured, whereas the C-terminal region is distinguished by its globular domain comprised of three α-helices interspersed by two double stranded anti-parallel β- sheets, followed by its GPI anchor at the very end (Aguzzi and Calella 2009). Ion pairing between α-helices, intramolecular binding between β-sheets, as well as a disulfide bridge between cysteine residues 179 and 214 act to stabilize the protein's secondary structure within the C-terminal. Conversely, the N-terminal region is considered disordered and flexible while lacking secondary structure. This region contains a central domain between residues 95 to 133, which is comprised of a charged cluster and hydrophobic core. The central domain's function is not entirely clear, but conformational study suggest it is the primary target of PrP^{Sc}-induced protein misfolding (Taraboulos, et al. 1990). It is likely that conformational change in the central domain reveals binding sites, specifically at residues 106-126, which promotes cytotoxicity by activating caspase-3, thus compromising the plasma membrane leading to apoptosis (Vilches, et al. 2013). Almost the entire N-terminal region sequence constitutes a basic motif , which is essential for transport of the protein through the secretory pathway to the plasma membrane as well as its internalization via association with the transmembrane low-density lipoprotein receptor-related protein 1 (LRP1) (Nunziante, et al. 2003; Parkyn, et al. 2008; Sunyach, et al. 2003). Recently it has been discovered that positively charged N-terminal

sequences at residues 23-31 and 95-105 can bind amyloid β (Aβ) oligomers, an association which has been postulated by different investigative groups to be necessary for or inhibits Aβ-mediated cytotoxicity (Fluharty, et al. 2013; Lauren, et al. 2009). And finally, the N-terminal contains five glycine-rich Octapeptide repeats (OR) from residues 51 to 90 that are involved with important functions to be discussed below (Hooper, et al. 2008). **See Figure 1.1** for PrP^C primary structure illustration.

Figure 1.1. PrP^C primary structure schematic. OR: Octapeptide Repeats; CC: Charged Cluster; HC: Hydrophobic Core; B1/2: β-sheets 1 and 2; H1/2/3: α-Helices 1, 2, and 3. Arrow at residue 23 indicates cleavage site of N-terminal signal peptide. Disulfide bond shown between residue 179 and 214. Carbohydrate moiety N-glycosylation sites at N-181 and N-197 positions.



1.3. Role

Given that PrP^C is likely expressed in all vertebrates and shares mostly conserved sequence and properties, it is within reason to consider that the protein plays a fundamental role. Animal studies using PrP^C-knockout strains of mice showed that phenotypically there was no gross disturbances in behavior and development (Bueler, et al. 1992; Manson, et al. 1994). A possible

explanation for this lack of change is that there are compensatory mechanisms that carry out PrP^C's functions. However, this suggestion is highly disputed as other studies provide evidence of PrP^C-knockout mice displaying altered behavior due to disrupted circadian rhythm (Tobler, et al. 1996), changes in electrophysiological signaling (Colling, et al. 1996), abnormal long-term potentiation and γ-Aminobutyric acid (GABA)-inhibition (; Collinge, et al. 1994) . Cell culture study shows that PrP^C-deficient neuronal lines are less viable and more susceptible to oxidative stress (Brown, et al. 1997b) and toxicity (Kuwahara, et al. 1999) compared to wild-type cells; . Due to PrP^C's high expression in the brain, especially between synapses, both pre- and post-synaptically, it is not surprising that the protein plays an important role in neural transmission (Collinge, et al. 1994; Haeberle, et al. 2000; Herms, et al. 1999; Prusiner 1982; Sales, et al. 1998). Examination of neuronal pathways within the hippocampus of PrP^C-knockout mice show reduced excitability in hippocampal granule cell layers (Colling, et al. 1996) as well as mossy fiber pathway reorganization, which promotes epileptiform activity (Colling, et al. 1997). Taken together, studies investigating PrP^C-deficiency at whole animal, tissue, and cellular levels indicate that the protein is highly involved in regulating the perineuronal environment. Discussed below are several functions of PrP^C implicated in neuronal homeostasis in addition to evidence that collectively suggests PrP^C plays a neuroprotective role in the CNS overall.

1.3.1. Metal Ion Binding

As aforementioned, the N-terminal half of PrP^C contains five OR segments consisting of one incomplete sequence (PQGGTWGQ) followed by four functional ones (PHGG(G/S)WGQ) (Sumudhu, et al. 2001). Within each functional repeat is a histidine residue capable of binding one Cu^{2+} ion. Binding affinity for one Cu^{2+} atom is low, but when binding multiple atoms the

affinity increases significantly suggesting the first binding event facilitates subsequent copper saturation. Structural binding analysis of the N-terminal encompassing the OR shows additional histidine residues capable of binding copper at residues 96 and 111; in total PrP^C can be saturated with 4-6 Cu^{2+} ions (Brown, et al. 1997a; Liu, et al. 2011). Upon binding to copper, the OR undergoes conformational changes adopting a loop-like and β-sheet structure (Zahn 2003). Copper-bound PrP^C then translocates laterally out from lipid rafts into detergent-soluble regions of the plasma membrane where it rapidly undergoes endocytosis via clathrin-coated pits (Hooper, et al. 2008). At physiological levels copper is an important element in CNS development, but when in excess as implicated in various neurological disorders, there may be production of free radicals leading to mitochondrial, DNA, and overall cell damage (Desai and Kaler 2008). Comparison of crude membranes, synaptosomes, and endosomes derived from PrP^C-knockout mouse brain show drastically reduced (80%) intracellular copper content compared with wild-type (Brown, et al. 1997a). Considering the importance of copper within the CNS along with evidence of impaired uptake with diminished PrP^C, the protein is likely a major contributor towards synaptic homeostasis. Besides copper, the metal binding regions within the PrP^C N-terminal are also able to bind other divalent metal cations such as nickel, zinc, iron, and manganese with decreasing affinity (Jackson, et al. 2001; Singh, et al. 2010).

In a similar manner to copper, PrP^C is also able to bind and facilitate Zn^{2+} uptake within cells through its OR region with lower binding affinity than copper (Pauly and Harris 1998; Perera and Hooper 2001; Watt, et al. 2012). Introduction of other metal ions such as manganese and iron elicited no direct PrP^C-mediated binding or internalization; however, PrP^C can alternatively affect intracellular iron content through an unidentified process (Pushie, et al.

2011). Upon cellular depolarisation, vesicle packaged zinc is released from the pre-synapse into the synaptic cleft, which is then taken up into the cytoplasm of the post-synaptic neuron via zinc transporters or activated voltage-gated calcium channels α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) receptors and N-methyl-D-aspartate (NMDA) receptors (Sensi, et al. 2009). PrP^{C} was found to mediate zinc uptake by interaction with GluA1- and GluA2-containing AMPA receptors (Watt, et al. 2012). Studies examining brains of animals with compromised PrP^{C}, due to mutation or PrP^{Sc} infection, shows that zinc uptake is disrupted. As zinc deficiency negatively affects neurogenesis and increases neuronal apoptosis while excess release damages post-synaptic neurons, PrP^{C} is important for maintaining this narrow homeostatic range (Plum, et al. 2010; Szewczyk 2013). Thus, PrP^{C} is involved in zinc sensing and scavenging required to maintain homeostasis, the dysregulation of which can be associated with neurodegenerative diseases such as Alzheimer's disease (AD).

1.3.2. Anti-oxidative properties

PrP^{C}-mediated endocytosis of copper was shown to be important for cellular Cu-Zn superoxide dismutase (SOD) activity, which indicates the protein is involved in oxidative stress resistance (Brown, et al. 1997a; Brown, et al. 1999; Pauly and Harris 1998; Perera and Hooper 2001). Evidence for this notion is seen in a study using cerebellar slice preparations from wild-type, PrP^{C}-deficient, and PrP^{C} gene-reconstituted transgenic mice (Herms, et al. 1999). Since hydrogen peroxide is decomposed to form highly reactive hydroxyl radicals when combined with excess synaptic concentrations of metal ion Cu^{2+}, which in turn damages and reduces synaptic activity, investigators determined that higher synaptic responses positively correlated with increasing expression of copper-sequestering presynaptic PrP^{C}. Additionally, PrP^{C}-deficient

cells display reduced SOD activity and are more susceptible to oxidative stress and copper toxicity (Brown, et al. 1998a; Brown, et al. 1997b). During oxidative stress there is a compensatory induction of anti-oxidative enzymes such as SOD, which apparently functions in concert with PrP^C. The copper binding site located on the OR region prevents the formation of reactive oxygen species by promoting the reduction of Cu^{2+} to Cu^+ to prevent excessive redox cycling and reactive oxygen species formation (Liu, et al. 2011). Additional evidence of PrP^C's anti-oxidative ability is that cells expressing PrP^C are more resistant against manganese-induced oxidative stress and apoptosis as compared to PrP^C-knockout mice (Choi, et al. 2007). Study using TSE-infected brain tissue shows loss of normal function of PrP^C due to misfolded plaque formation resulting in depleted copper-dependant anti-oxidative activity as well as manganese-induced oxidative stress (Johnson, et al. 2013). Thus, PrP^C plays a neuroprotective role by promoting ion trafficking and homeostasis within the extracellular micro-environment.

1.3.3. Excitotoxicity Modulation

Of all the cell surface protein interactions with PrP^C, the most intriguing and perhaps well studied over the past decade has been with glutamate receptors, both metabotropic and ionotropic. The three classes of ionotropic receptors, AMPA, kainate, and NMDAR have been shown to interact with PrP^C (Beraldo, et al. 2011; Carulla, et al. 2011; Kleene, et al. 2007; Mayer 2005; Um, et al. 2013; Watt, et al. 2012). A study using PrP^C-deficient mice found the animals displayed increased NMDAR-dependent excitability and subsequent excitotoxicity due to lost attenuation of PrP^C on NR2D subunits (Khosravani, et al. 2008). Most recently, interaction between PrP^C and NR2B subunits of NMDR has been demonstrated, which may have a role in attenuating NR2B-dependent excitatory function of NMDARs (Pham, et al. 2014). Furthermore,

in the brain of a pre-diabetic animal model this interaction is compromised due to PrP^C suppression combined with overexpression of NR2B subunit protein compared with normal wild-type brains. Excessive NMDAR activity mediates increased Ca^{2+} entry into neuronal cells, thus contributing to excitotoxic death. Studies utilizing animal stroke models showed that there was a time-dependent upregulation in expression of PrP^C in ischemic brain tissue as well as the penumbric region following focal ischemia compared to untreated brains (Mitsios, et al. 2007; Shyu, et al. 2005). Moreover, the upregulated PrP^C was shown to colocalize with neuron, glia, and vascular endothelial cells likely reflecting a broad adaptive response to ischemia and hypoxic conditions. This is apparent as evidenced by transgenic animal studies showing overexpression of PrP^C reduces volume of cerebral infarction (Shyu, et al. 2005) compared with PrP^C-deficient mice whose neuronal cells are also susceptible to NMDA-dependent cell death (Spudich, et al. 2005). This evidence supports the notion of PrP^C's adaptive response role as purported in an earlier animal stroke study, which showed upregulation of PrP^C within the ischemic hemisphere (Weise, et al. 2004). This effect was most significant in the acute period following insult, the duration of which depended on the extent of neuronal damage. Clinical study using stroke patients provided evidence that PrP^C expression is increased within the peri-infarcted brain tissue in the neuronal soma, endothelial cells of micro-vessels, and pro-inflammatory immune cells (Mitsios, et al. 2007). Increased PrP^C within the peri-infarcted region may influence hypoxia-induced cell damage, the association of which has been recently confirmed in animal studies showing that hypoxia-inducible factor-1 α can regulate PrP^C expression and protects against neuronal damage (McLennan, et al. 2004). Since NMDA activation can occur as a result of ischemia/hypoxia, it is likely that upregulation of PrP^C is necessary to attenuate receptor activity to prevent excitotoxicity. This association of PrP^C and

NMDA is particularly intriguing as various neurological conditions and disorders involve NMDAR over-activation such as ischemic stroke, traumatic brain injury, AD, Huntington's disease, and epilepsy (Parsons and Raymond 2014).

In summary, PrP^C is a unique endogenous protein mainly expressed within the CNS that plays a role in several important physiological functions, including neuroprotection. If increased PrP^C levels within the brain are indicative of an adaptive response, then perhaps assessing its levels within biological fluids can also be telling.

Section 2. A biomarker within the blood: PrP^C

2.1. PrP^C in Plasma

Using flow cytometry, investigators have discovered that PrP^C is differentially expressed on the surface of various blood formed elements. Mononuclear leucocyte and platelet have the highest surface expression of PrP^C, while polymorphonuclear leucocytes and red blood cells (RBCs) express little to no PrP^C (Cashman, et al. 1990; Dodelet and Cashman 1998; Vostal, et al. 2001). The source of PrP^C within blood cells can be traced to within the bone marrow where it is expressed on all long-term hematopoietic stem cells, and are required for maintaining self-renewal (Zhang, et al. 2006). Following hematopoiesis, platelet-derived PrP^C accounts for the majority of cell-expressed PrP^C in whole blood. Multiple studies have shown that following platelet activation, there is a drastic increase in PrP^C expression compared to resting cells (Vostal, et al. 2001). Additionally, upon stimulation of platelet aggregation and adhesion, soluble PrP^C is released from the plasma membrane (Perini, et al. 1996). In contrast, RBCs express approximately four fold less PrP^C than platelets, but due to their quantity outnumbering platelets by twenty fold they are a greater contributor towards blood PrP^C levels. However, there is no definitive consensus upon the contribution of PrP^C by these fractions based on varying detection sensitivity across studies (Panigaj, et al. 2011; Vostal, et al. 2001). Endothelial cells, another contributor towards constitutive plasma PrP^C levels, have been shown to release PrP^C expressed in microparticles, such as exosomes, during apoptosis (Simak, et al. 2002). Thus endothelium-derived PrP^C within the plasma is likely a result of regular cell turnover of the vascular lining.

The act of using prion protein in blood as a biomarker has only recently become prominent due to cases of iatrogenic transmission of variant CJD via blood transfusion arose in

Great Britain (Wroe, et al. 2006). Biological fluids and tissue from TSE-infected animals, such as cows with BSE, carry a high potential to spread the disease to other animals including humans. In animal studies, transmission was demonstrated using blood from infected sheep that were symptomatic of scrapies, as well as those within the asymptomatic preclinical phase (Houston, et al. 2000; Houston, et al. 2008). Furthermore, transmission was demonstrated after transfusion of cellular blood elements, plasma as well as the cryoprecipitate fraction of plasma (Brown, et al. 1998b). Knowing that blood cells express PrP^{C} on their surface combined with the fact that the pathogenic misfolded PrP^{C} isoform acts as a template for conformational conversion of normal protein into nascent PrP^{Sc}, clearly the infectious potential of TSEs can be carried within blood. The earliest methods for detecting TSE-causing PrP^{Sc} relied on post-mortem testing of brain tissue, but testing for its presence in biological fluids such as blood remained difficult. Validation of blood screening procedures required testing on thousands of healthy samples in addition to CJD-positive ones.

2.2. PrP^{C} as a Biomarker

Early screening procedures focused on qualitative detection of only toxic prions because quantification for PrP^{C} was considered to have little clinical value in detecting disease. However, an under-appreciated report by Völkel and coworkers had emerged years earlier in which they quantified plasma PrP^{C} as a biomarker for a broad spectrum of neurologic diseases including AD, Parkinson's disease (PD), and dementia, with a particular emphasis on CJD (Völkel, et al. 2001). Using enzyme linked immunosorbent assay (ELISA) techniques, Völkel's group found that plasma PrP^{C} was elevated in patients with neurodegenerative diseases when compared with healthy control samples. Intriguingly, elevated plasma prions from CJD patients

was protease K sensitive and thus the non-pathologic isoform, which is counter intuitive to past observations of increased proteinase-resistant PrP^{Sc} in CJD cases (Wroe, et al. 2006). It is possible that PrP^{C} expressed on the surface of components of the blood such as T and B lymphocytes as well as platelets are released when the cells become activated in such disease states (Halliday, et al. 2005; MacGregor, et al. 1999). Another possibility is that PrP^{Sc} infection in the brain results in upregulation of PrP^{C} as an adaptive response against glutaminergic excitotoxicity in neighboring cells, due to significant degeneration and cell damage, subsequently leading to increased protein shedding from the CNS (Ankarcrona, et al. 1995). To show PrP^{C} is capable of crossing the blood brain barrier (BBB) and entering the bloodstream from the CNS, Banks and coworkers used radiolabeled PrP^{C} to demonstrate bi-directional transport from the systemic circulation to the brain tissue and from the cerebrospinal fluid (CSF) to peripheral tissues with varying region-specific tissue uptake (Banks, et al. 2009). They further postulated that PrP^{C} levels are maintained in a steady state equilibrium across the BBB, and that perhaps elevated concentration within the circulation is a response to certain pathological or neurodegenerative states.

More recent studies have investigated using soluble PrP^{C} as a biomarker for human immunodeficiency virus (HIV)-associated neurocognitive disorders (HAND) (Megra, et al. 2013; Roberts, et al. 2010). These studies examined HIV-1 infected individuals with HAND, dementia, encephalitis, or without neurological impairment, as well as a comparable animal model of simian immunodeficiency virus (SIV)-infected macaques with encephalitis. In HIV-1 infected individuals with HAND, dementia, and encephalitis, as well as SIV-1 infected animals, there was evidence of increased PrP^{C} expression within astrocytes and neurons along with

elevated CSF concentration, which positively correlated to the degree of neurological impairment or encephalitis when compared against non-infected controls and those infected without impairment (Roberts, et al. 2010). Plasma PrP^C concentration was also evaluated which, as opposed to CSF levels, were decreased in all HIV-infected individuals compared to healthy controls. It is noteworthy that their study provides little information describing characteristics of the human controls included nor did they assay a sufficient number (n=3) of normal samples to draw conclusions from. Conversely, their animal experiments show that PrP^C was persistently elevated within the plasma at multiple time points post-infection (7-56 days) compared to controls. The authors do not speculate on these opposing findings, but one must consider that their animal experiments were well controlled for genetic and environmental influences as opposed to a relatively diverse pool of humans. Moreover, animal blood samples were taken relatively early compared to human participants who may have had HIV for years prior to the study. Taken together, their evidence suggests that PrP^C is a biomarker of neurodegeneration, which becomes upregulated in the brain and subsequently secreted or shed into biological fluids.. However, this assumption may also work in the opposite manner as another study showed, using a larger sample set, that CSF PrP^C levels are decreased in various neurodegenerative conditions or diseases compared to control (Meyne, et al. 2009). Similarly, PrP^C within human CSF was found to decrease to near undetectable levels, without apparent PrP^C breakdown products, one week after individuals suffered spinal cord injury suggesting CNS-specific uptake of the protein, perhaps for neuroprotection (Carnini, et al. 2010).

Another potential means of increased plasma PrP^C from the CNS was discovered when investigating calpains, which are a class of ubiquitously expressed cytosolic non-lysosomal

cysteine proteases that require calcium to function. When there is excessive glutamate-mediated influx of calcium into the neuron, such as following a cerebrovascular insult or even traumatic brain injury (TBI), hyperactivation of available calpains occurs allowing for unregulated proteolysis of both specific and non-specific proteins leading to tissue damage (Liu, et al. 2008). The resulting proteolytic cleavage products from increased calpain activity can be used as biomarkers for the presence of neurological degeneration or injury. However, when calpains are introduced to brain lysates from PrP^C-expressing mice there is surprisingly no appearance of PrP^C breakdown products, despite the protein's well known susceptibility to proteases (Wang, et al. 2012). This same study also showed that after neurotoxic treatment with subacute doses of maitotoxin and NMDA resulting in calcium influx, breakdown products are still not detected. But instead of smaller fragmented protein products, they found that full-length intact PrP^C molecules were actually released into the culture media, in a toxicity dependent manner, via a process completely independent of calpain and other cytosolic proteases. This indicates that, by some yet unidentified cleavage event, full-length PrP^C is released as a result of cell stress.

Another source for plasma PrP^C can be found in microvesicular exosomes isolated from plasma, which express PrP^C (Ritchie, et al. 2013). Moreover, cultured neurons have been shown to release PrP^C-carrying exosomes into the surrounding media, which is likely a means of maintaining homeostasis *in vivo* as mentioned above (Fevrier, et al. 2004). Exosomes are extremely small cell-derived microvesicles (30-100 nm) that are formed when internal multivesicular bodies fuse with the plasma membrane and then secreted into the extracellular environment (Keller, et al. 2006). Exosomes can be derived from many different cell types including immune cells, platelets, and neurons and are detectable within biological fluids such as

blood plasma (Caby, et al. 2005). Exosomes can transfer molecules, proteins, metabolites, and genetic material from one cell to another via membrane vesicle trafficking without requiring direct cell contact or axonal transport. In this way, exosomes have been reported to be involved in cellular functions including protein secretion, RNA and protein transfer, cell-to-cell signaling, and even in neuroinflammatory responses, such as following TBI (Gupta and Pulliam 2014; Sharma, et al. 2013). Exosomes thus have the potential to affect cells up to long distances from their origin as they are transferred in biological fluids like plasma, and may be used as miniature surrogates of their origin cell's status upon secretion. For example, cultured neurons following PrP^{Sc} infection secrete exosomes carrying both PrP^{C} and PrP^{Sc} into the surrounding media (Fevrier, et al. 2004). Another scenario for exosome shuttling and release of PrP^{C} within plasma may be a result of a disintegrin and metalloprotease (ADAM)10. ADAM10 can act at the cell surface to cleave other surface proteins including PrP^{C} (Altmeppen, et al. 2011). Upon calcium influx, such as from stroke or TBI, there is augmented release of functional ADAM10-containing exosomes that is believed to be the primary contributor towards ectodomain shedding of soluble molecules, perhaps even PrP^{C} (Stoeck, et al. 2006).

There is a great deal of evidence describing the possible sources of soluble PrP^{C} within blood plasma. There seems to be a constitutive release and even uptake of soluble PrP^{C} within the CNS that is normally a part of its homeostatic regulation. However, in neurodegenerative states there is likely increased PrP^{C} release from the CNS into the periphery. Such conditions have garnered much attention in particular as it pertains to TBI survivors that may develop long-term neurological deficits.

Section 3. Traumatic Brain Injury (TBI)

Traumatic brain injury (TBI) is the result of external mechanical forces exerted on the brain as a result of direct or indirect physical impact to the body. Direct physical TBI can occur as a result of falls, vehicle accidents, assaults, and sports, which will be of particular focus from here on, may be a result of impact to the head, face, neck, or other parts of the body by a solid surface or object, or even another person. This form of TBI involves sudden linear or angular acceleration/deceleration forces that are known to cause discrete, focal lesions at points of impact, shearing stress on the brain, and BBB disruption (Marchi, et al. 2013; Patel and Reddy 2010). Conversely, indirect TBI can occur as a result of transferred kinetic energy such as from exposure to explosive blast waves commonly experienced by military service members. Unlike conventional head injuries such as in sports, blast-induced TBI (bTBI) produces no linear or angular forces as the necessary whiplash or jolting motion of the head are absent without involvement of the cervical spine. Instead the mechanism of injury for bTBI involves various components of the blast contributing towards a heterogeneous pathology complex. Although both forms of TBI may seem grossly different from one another, in actuality they may be very similar as most head injuries are mild in severity with most of the brain damage occurring as a result of secondary injury mechanisms being active for an extended period of time after the initial insult. Athletes and military service members who have suffered multiple mild-TBI display altered mental status; impaired cognitive, motor, and social function; increased risk for AD, amyotrophic lateral sclerosis (ALS), PD, and chronic traumatic encephalopathy (CTE)-like symptoms (Goldstein, et al. 2012; McKee, et al. 2009; McKee and Robinson 2014; Omalu, et al. 2005). TBI is a growing epidemic because victims of sports- or blast exposure-TBI may not be

accurately identified as injured by clinicians perhaps due to insufficient diagnostic test results or even under-reporting of symptoms by those injured.

3.1. Sports Concussions

Concussion is a complex pathophysiological process and is considered as a subset of mild TBI (mTBI), which results in transient disturbances of brain function as a result of head injury. Concussions are the consequence of a direct or indirect blow that results in a sudden angular acceleration or deceleration of the brain tissue within the calvarium. In the US alone, 3.8 million cases of sport-related concussions occur annually, and high-contact sports such as American football, hockey, rugby, soccer, and basketball have the highest incidence of concussion (Daneshvar, et al. 2011; Harmon, et al. 2013; Langlois, et al. 2006; Meehan and Micheli 2011). Considering unreported cases by victims, it is estimated that the incidence of sport-related concussions is even higher (Meehan, et al. 2013). Clinical manifestations of sport-related concussions may include a variety of symptoms such as loss of consciousness, headache, dizziness, amnesia, nausea, confusion, fatigue, sleep disturbances, balance and memory impairment, slurred speech, and light sensitivity. At the molecular pathophysiological levels, most of these symptoms are a result of significant alterations in ionic balance, neurotransmitter activation, axonal integrity, and energy metabolism in the CNS (Barkhoudarian, et al. 2011; McKee, et al. 2014). Most sport-related concussions are benign and athletes typically recover within 7-10 days or even longer if given adequate rest and appropriate therapy. During this sensitive period of recovery, individuals are extremely at risk should they suffer a subsequent head injury. Multiple concussions within a short period of time may lead to devastating long-term sequelae and prolonged functional impairment, including post-concussive syndrome,

neurodegenerative diseases, CTE, as well as rare catastrophic consequences called second impact syndrome (Boden, et al. 2007; Gavett, et al. 2011; Halstead and Walter 2010; Omalu, et al. 2005).

In high-contact sports it is not uncommon for athletes to suffer multiple head injuries resulting in concussion, but they may also suffer many more sub-concussive impacts that can potentially compile over time with worsening neurological degeneration. An animal model of repetitive mTBI showed persistent mild deficit in balance and coordination, increased expression of axonal injury marker glial fibrillary acidic protein (GFAP), as well as hyperphosphorylation of tau, a cellular microtubule-associated protein (Kane, et al. 2012). In humans and animal models of TBI, there is evidence of microvasculature and BBB damage, cerebral hemorrhaging, neuroinflammation, edema, oxidative stress, ischemia, cell death, and diffuse axonal injury (Foda and Marmarou 1994; Fritz, et al. 2005; Homsi, et al. 2009; Marmarou, et al. 1994). There is even evidence of acute elevation in AD-associated Aβ40 and 42 peptides as well as increased levels of β-site APP cleaving enzyme 1 (BACE1) and γ-secretase component presenilin-1 (PSEN1) (Loane, et al. 2009). This shift in normal APP processing towards deleterious peptide formation combined with widespread tau phosphorylation shows there is an associative risk between brain injury and AD as shown by amyloid β deposition within axons as early as a few days following a TBI event (Johnson, et al. 2012; Smith, et al. 2003). When combined, evidence from TBI animal models and gross pathology of post-mortem brains from athletes who have suffered repetitive TBI suggests that there are a myriad of secondary injury mechanisms occurring over time that contribute towards progressive neurodegeneration like CTE, and increased risk for diseases, such as AD, PD, and ALS (Omalu, et al. 2005). Since secondary

injury mechanisms are likely occurring at the cellular level, it is not surprising that most mTBI cases show no abnormalities on computed tomography (CT) and conventional magnetic resonance imaging (MRI). Identifying those athletes affected by concussion remains a challenging issue for health care clinicians using current standards of care (Belanger, et al. 2007). Thus, it is absolutely essential to identify whether concussion occurred so as to manage it properly and avoid repetitive injuries.

3.2. Blast-induced TBI

Blast-induced TBI (bTBI) is the result of explosive shock waves impacting the body. This is different from conventional impact-related TBI as blast waves directly transfer kinetic energy throughout the body while the former requires an acceleratory force component. Although bTBI primarily affects military personnel (to be discussed further in published work below in Section 5), it is also of concern for civilians exposed to blasts, such as in industrial accidents or urban terrorism. Upon detonation of an explosive weapon or material, a large shock wave (primary blast wave) is released due to instantaneous conversion of stable material into gas that expands outwards from the blast epicenter. As the primary blast wave traverses in air, there is increased atmospheric pressure, termed the blast overpressure (BOP) wave, as a result of air displacement in front of the wave. Conversely, this overpressure wave is shortly followed by a pressure front below normal atmospheric level, termed the underpressure wave, which then equilibrates back to normal as displaced air is replaced. Molecular and cellular mechanisms of brain injury following BOP are not well understood and difficult to study due to the heterogeneity of contributing blast by-products including the overpressure wave, heat, and chemicals. This thesis will only discuss injury mechanisms related to BOP.

When the BOP wave makes contact with the body, not necessarily just the head, there is a transient surge in atmospheric pressure within hollow organs and the cardiovascular system (Chen, et al. 2013). This increase in overall pressure then rapidly causes an adaptive physiological increase in blood pressure to displace blood and return to normal pressure levels. The sudden surge of displaced blood is likely directed towards the cranial cavity as a result of it being lower in pressure as the skull is able to resist atmospheric compression from the BOP wave unlike the rest of the body. Blood surging through the brain results in large-scale microvasculature and BBB damage, subsequently leading to activation of secondary injury mechanisms (Chen, et al. 2013). Additionally, gas filled spaces such as the lung, ear, and gastrointestinal tract are commonly injured following blast exposure due to sudden atmospheric constriction and subsequent implosion of compressed gases (Mayorga 1997). Violent displacement of fluids and gases within the body following blast-exposure can result in shearing or tearing forces due to the blast wave-induced acceleration of tissue at varying rates depending on their density (Nakagawa, et al. 2011).

Animal study has shown that similar to mTBI, blast exposure produces very similar pathology as conventional TBI experiments. Rats exposed to BOP revealed significant increase in reactive oxygen species, altered anti-oxidative enzyme activity, as well as expression of pro-inflammatory mediators (Cernak, et al. 2001; Cho, et al. 2013). This environment of oxidative stress and neuroinflammation resulted in neuronal cell death as well as significantly impaired short-term memory and learning. BOP studies using animals and organotypic brain slice culture have shown that even mild intensity shock waves can cause hippocampal cell death, neuronal, glial, and vascular damage, astrogliosis, axonal deposition of Aβ, as well as altered cell

attachment and signalling (de Lanerolle, et al. 2011; Effgen, et al. 2014; Kamnaksh, et al. 2012). However, in contrast to non-blast TBI, blast exposure actually results in decreased brain levels of Aβ 40 and 42 with no change in APP processing secretases (De Gasperi, et al. 2012). Although there is evidence of the development of CTE-like pathology after a single blast event in an animal BOP model as well as in war veterans exposed to multiple blasts, there has been no definitive link to developing AD besides the risk from shared pathology of neurofibrillary tau tangles (Goldstein, et al. 2012).

Overlapping symptoms with conventional TBI as well as psychological conditions such as post-traumatic stress disorder (PTSD), including sleep disturbances, headaches, nausea, and memory loss, can produce confounding effects on diagnosis (Weinberger 2011). In the military, misdiagnosis of blast exposure is very possible considering the constant duress combined with physical and psychological strain and trauma soldiers may face in duty that these symptoms can arise from. Furthermore, under-reporting of symptoms such as headaches, dizziness, or memory loss by soldiers is common in part because of traditional militaristic zeal to endure one's pain and hopefully "shrug it off". As a result, military service members are especially at risk for repeated blast exposure during missions as many are not given medical exemption for duty in comparison to those that received penetrating wounds for example.

3.3. Protein Biomarkers of TBI

Diagnosis of TBI is especially difficult because of the heterogeneous manifestation of signs and symptoms with low reproducibility across different cases. Current standards of diagnosis for TBI are not definitive, but are usually inferred by clinicians based on cognitive

impairment tests such as the Glasgow Coma Scale (GCS), which requires clinicians to score individuals based on ocular, verbal, and motor responses. As a result, researchers have focused on screening for objective surrogate protein biomarkers specific to TBI. Protein biomarkers are readily accessible in biological fluids such as plasma and serum, which may serve as valuable tools in identifying those who have suffered TBI. Several potential protein biomarkers have been identified for TBI, of which a few have been tested in sport related concussion (reviewed in (Jeter, et al. 2013; Strathmann, et al. 2014; Yokobori, et al. 2013). Among these potential protein biomarkers, S100 calcium binding protein B (S100B), cleaved and total tau, GFAP, myelin-basic protein (MBP), neuron-specific enolase (NSE), ubiquitin C-terminal hydrolase-L1(UCH-L1), αII-spectrin breakdown products (SBDPs), 14-3-3 (Siman, et al. 2009), apolipoprotein E (apoE) (Kumar, et al. 2006), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α) have been widely studied for their ability to detect TBI in both sports-related and blast exposure injuries. However, there is no one ideal biomarker to determine TBI as many studies provide conflicting evidence promoting or refuting the use of certain proteins. One of the most widely investigated proteins S100B, an astroglial injury marker, is increased in the blood following head trauma, but it can also be increased as a result of extra-cranial injuries and bone fractures (Anderson, et al. 2001; Vos, et al. 2010). In contrast, another clinical study found that plasma S100B levels did not correlate with TBI in head-injury patients upon hospital admission (Metting, et al. 2012). However, the same study showed that plasma GFAP was highly specific for TBI, but showed poor sensitivity as it was indiscernible in more than half the cases. A multicenter cohort study following professional hockey players showed that plasma levels of total tau and S100B were significantly elevated in players one hour following concussion compared to their pre-season values, while NSE showed no change (Shahim, et al. 2014). Repeated blood sampling at set

intervals from 1-144 hours after injury showed that protein levels for T-tau and S100B were elevated one hour following concussion and diminished greatly within 12 hours, with S100B becoming indistinguishable to preseason levels. However, the authors also note that following a friendly game without contact or concussion there was a significant rise in these proteins compared to levels taken before the game suggesting T-tau and S100B are non-specific to TBI. In contrast to this cohort of hockey players, an animal BOP study examining plasma biomarkers two hours following blast exposure showed that plasma protein levels of NSE were significantly elevated in rats receiving multiple shocks compared to sham controls, but GFAP was not (Kamnaksh, et al. 2012). The lack of objective protein markers for TBI should be expected to some extent considering the diverse nature of TBI manifestation, and remains a challenge that delays acute diagnosis and timely decision making regarding course of care for the injured.

3.4. TBI implications towards PrP^C

Due to PrP^C's extracellular orientation, it is possible that during a concussive event, linear and/or rotational forces transmitted through the brain may cause damage resulting in the tenuously bound PrP^C to become dislodged and collect within the systemic circulation in the acute period following injury. Another factor that could potentially increase the amount of free PrP^C to collect within the bloodstream is that TBI can cause BBB damage allowing for increased permeability. An animal model of TBI found that PrP^C-deficient mice manifested larger lesion volume as well as more extensive BBB damage compared to wild-type controls (Hoshino, et al. 2003). As PrP^C is expressed within endothelial cell junctional domains of the BBB, it likely serves a protective function, as suggested by the previously mentioned animal study, and may be upregulated in response to TBI-induced damage (Viegas, et al. 2006). Following this acute

period of potential shedding, it is likely that PrP^C is upregulated within and surrounding damaged areas of the brain in which secondary injury mechanisms are active such as oxidative stress and inflammation. An animal BOP model showed that 24 hours following blast exposure *Prnp* is one of many upregulated genes within the brain (Kochanek, et al. 2013). Unfortunately due to the investigators' study design, both *Prnp* mRNA and its protein product did not meet the upregulation criteria for robustness as some other classical injury markers did, and was not further explored. Additionally, since neuronal excitotoxicity plays a major role in the pathogenesis of TBI, it is possible that PrP^C becomes upregulated, especially within synapses where it can modulate overactive NMDAR following injury (Algattas and Huang 2014; Parsons and Raymond 2014). As discussed in Section 1, an animal stroke model using transgenic mice for different levels of PrP^C expression showed that PrP^C serves a neuroprotective role in limiting brain infarction size following ischemia (Spudich, et al. 2005). Spudich and coworker's examination of affected cell signaling pathways revealed that PrP^C modulates activity of integral extracellular signal-regulated kinase (ERK)1/2, signal transducers and activators of transcription (STAT)1, and the pro-apoptotic enzyme caspase-3. Intracellular ERK1/2 signaling is highly active following TBI as shown by increased phosphorylation in mossy fibers of the hippocampus, and is thought to be involved with mossy fiber reorganization, which may pose a risk of seizure for those injured (Hu, et al. 2004). Since PrP^C-knockout mice also show mossy fiber network changes, the protein is likely involved in regulating ERK1/2 signaling as a neuroprotective measure against epileptiform activity (Colling, et al. 1997). STAT1 is another protein that shows increased activity, via phosphorylation and nuclear translocation, following neuronal injury such as from ischemia (Takagi, et al. 2002) or TBI (Zhao, et al. 2011). As STAT1 overactivation may contribute to neuronal injury through regulating phosphorylation and

transcription of proteins related to apoptosis, it would seem that PrP^C-mediated modulation of STAT1 is neuroprotective. Finally, caspase-3 is a pro-apoptotic enzyme normally activated during programmed cell death signaling and is increased following TBI, thus contributing towards brain tissue loss and potentiates signal cascades towards cell death (Clark, et al. 2000). Although previous report suggests PrP^C can down regulate capsase-3 activity (Spudich, et al. 2005), there is conflicting evidence showing overexpression of PrP^C triggers increased capsase-3 action (Paitel, et al. 2002). Paitel and coworkers demonstrated that proteasome inhibition, thereby increasing cell surface PrP^C localization, potentiates PrP^C-mediated caspase-3 activation while antibody sequestering of PrP^C at the surface prevented it. A possible consideration is that normal PrP^C expression basally inhibits or is insufficient to activate caspase-3. However, upon upregulation of PrP^C, as seen in various models of neuronal injury, an alternate signaling pathway may become active or PrP^C-signaling itself is now sufficient to activate caspase-3. Further speculation may point towards a compensatory cellular mechanism that triggers for shedding upregulated PrP^C from the cell surface to prevent excess caspase-3 activation. Together, the evidence of PrP^C's neuroprotective role in TBI and other neuronal injury models suggest a concomitant damage-dependent upregulation of PrP^C as an adaptive neuroprotective response.

Other secondary injury mechanisms may likewise influence PrP^C upregulation such as inflammation. Neuroinflammatory mediator chemokine CC ligand (CCL)2, expressed in various cell types including neurons, astrocytes, and microglia, is elevated in the CNS following TBI and is implicated in progressive secondary brain injury (Semple, et al. 2010). Introduction of recombinant CCL2 to cultured neurons has been shown to increase PrP^C release within

surrounding culture media (Roberts, et al. 2010). When this soluble PrP^C-rich media is introduced to astrocyte culture, there is increased production of inflammatory mediators CCL2 and interleukin-6 suggesting that PrP^C can potentiate the neuroinflammatory state following injury. However, others have reported that PrP^C acts to attenuate T-cell dependent neuroinflammation (Tsutsui, et al. 2008). Thus, more investigation is required to determine PrP^C's specific role regarding inflammation. As mentioned in section 2, PrP^C levels between the brain and extracellular environment are homeostatically maintained. Synaptic membrane bound α-secretase member ADAM10 has been reported as the primary sheddase of PrP^C *in vivo* (Altmeppen, et al. 2011). Shortly following TBI, ADAM10 is active during synaptic reorganization (Warren, et al. 2012), which may allow increased cleavage of upregulated PrP^C at its shared lipid raft domains. Another possible route for increased PrP^C within the circulation may be in exosomes as discussed in section 2 above (Fevrier, et al. 2004; Ritchie, et al. 2013). An interesting property of exosomes is their ability to horizontally delivery proteins, metabolites, as well as mRNA and miRNA to respectively allow *de novo* protein synthesis or modulation in the recipient cell (Valadi, et al. 2007). The transfer of genetic material through exosomes has been shown to influence survival of nearby cells through initiation of adaptive responses to changes in their surrounding environment such as from TBI (Redell, et al. 2010). Thus, upregulation of PrP^C in and surrounding damaged areas of the brain, combined with increased Ca^{2+} trafficking as mentioned in section 2, may prompt increased exosomal packaging of cell surface proteins such as PrP^C and its sheddase ADAM10 (Stoeck, et al. 2006). It is unclear whether this cascade of events influences nearby cells in a neuroprotective manner and shall be a point of future study, but it does provide another possibility for PrP^C release into the periphery from the CNS following TBI. See below figure 3.1 for schematic diagram of possible PrP^C

cleavage products and figure 3.2 for scenario diagram of possible TBI-mediated release mechanisms for PrP^C.

All evidence considered regarding PrP^C's structure, role, and cleavage mechanisms, we postulate that TBI, either from conventional impact and acceleratory forces or oscillating blast waves, may cause shedding of PrP^C from the CNS into the extracellular space and pooling within the systemic circulation. Furthermore, the cascade of secondary injury mechanisms initiated after injury may further promote PrP^C shedding. By utilizing sensitive quantification technique, we can determine the presence of injury by assessing plasma PrP^C as a biomarker.

Figure 3.1. Possible breakdown products of PrP^C following TBI. β-site cleavage between residue 90/91 due to reactive oxygen species (ROS) produces the N2 terminal fragment. α cleavage between 109/110 due to ADAM10 produces the N1 fragment. β cleavage and α cleavage at site 228/229 or breakage of the GPI anchor produces the C2 fragment. α cleavage at site 109/110 and cleavage at 228/229 or GPI breakage produces the C1 fragment. α cleavage at site 228/229 or GPI breakage procures full-length fragment.

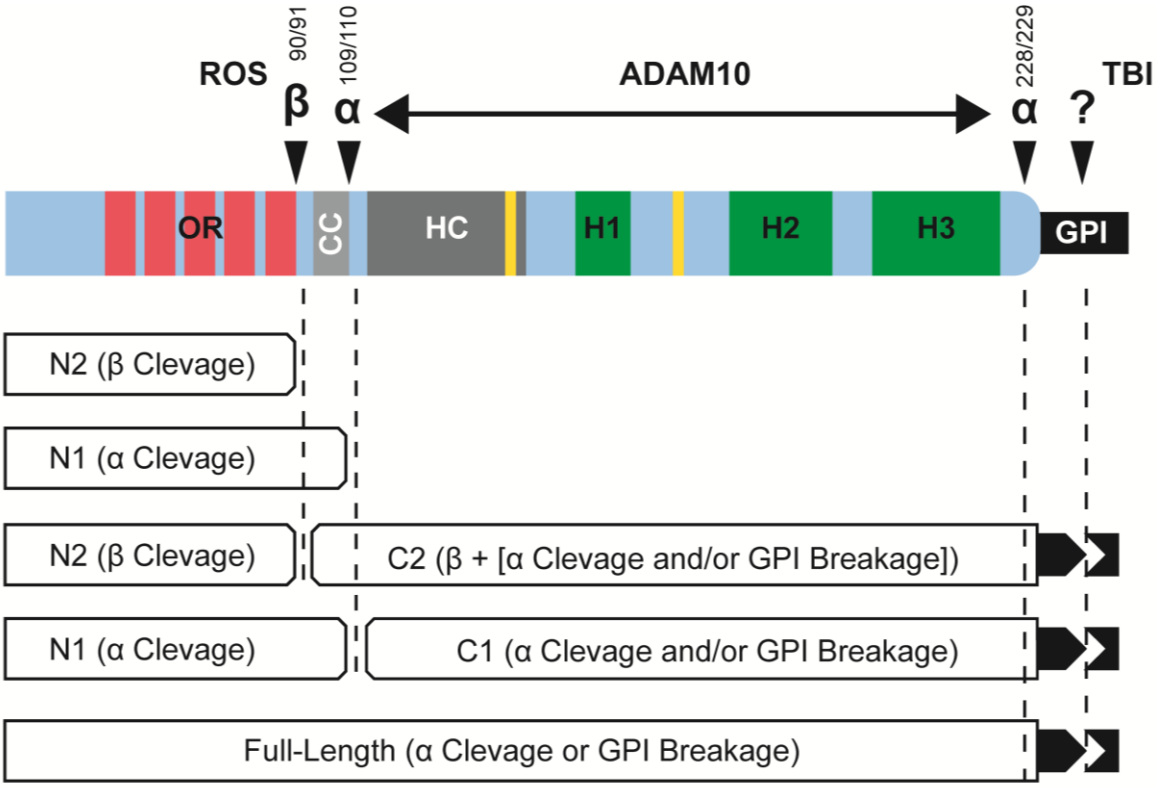
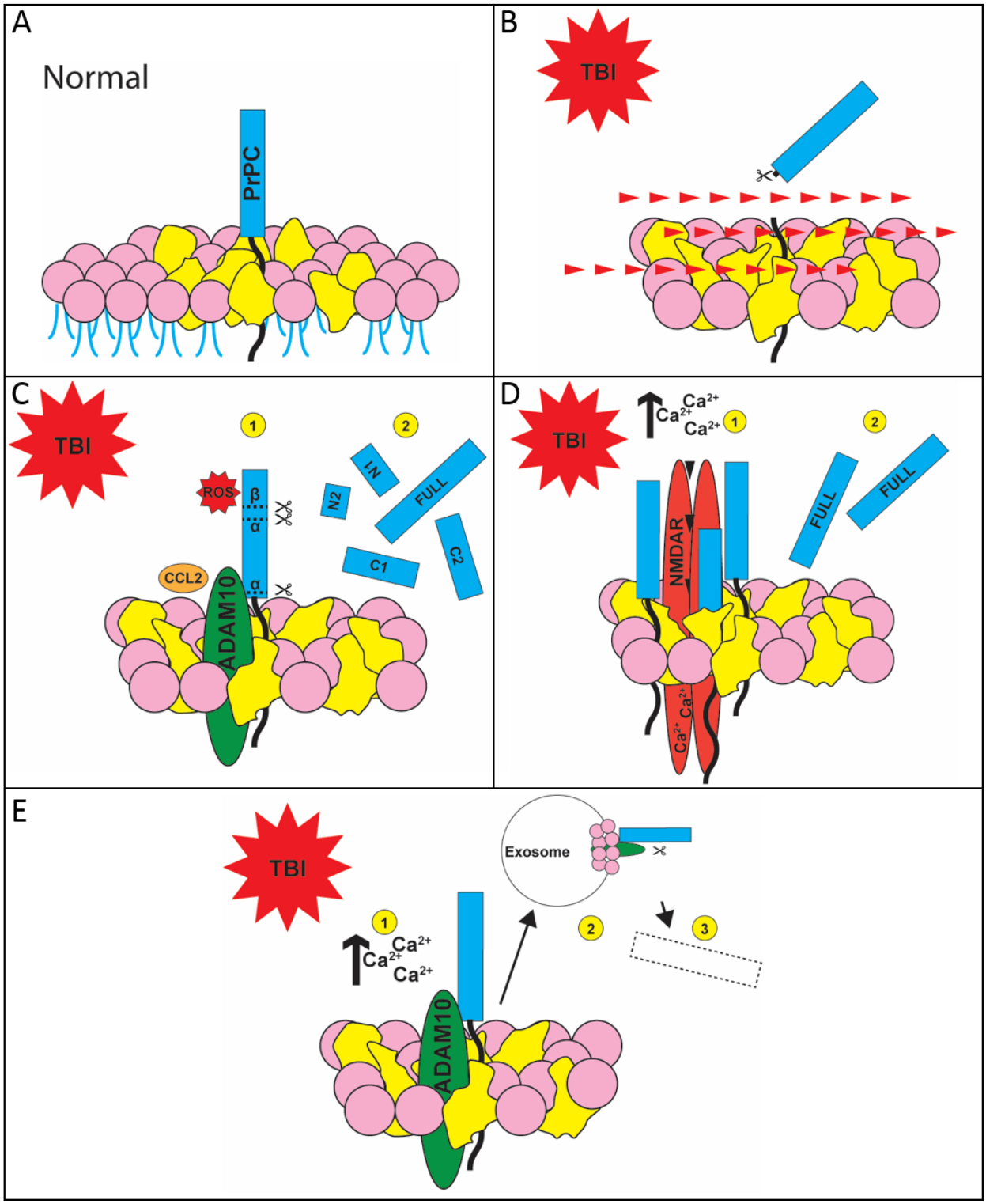


Figure 3.2. Possible release mechanisms for PrP^C following TBI. (A) Normal state with PrP^C attachment along extracellular surface of cholesterol-rich lipid rafts within the plasma membrane; **(B)** Shear stress force from TBI or oscillating waves from bTBI may cause breakage of the GPI anchor leading to free PrP^C within the extracellular space; **(C)** 1) Active ADAM10, elevated reactive oxygen species (ROS) and CCL2 can all contribute towards 2) PrP^C cleavage of various sized fragments; **(D)** 1) Elevated Ca^{2+} influx through overactive NMDAR contributes towards neurotoxicity. Increased expression of PrP^C at the cell surface acts to attenuate NMDAR activity resulting in 2) concomitant secretion; **(E)** 1) Elevated Ca^{2+} influx causes exosomal packaging of plasma membrane segments including functional ADAM10 and PrP^C. 2) Extravesicular shedding of PrP^C by ADAM10 may occur within the extracellular space leading to 3) its secretion (dotted). *Note: only the outermost layer of the plasma membrane (pink) is depicted. Lipid raft regions are indicated by cholesterol (yellow) rich areas within the plasma membrane.*

Figure 3.2. continued



Section 4. Materials & Methods

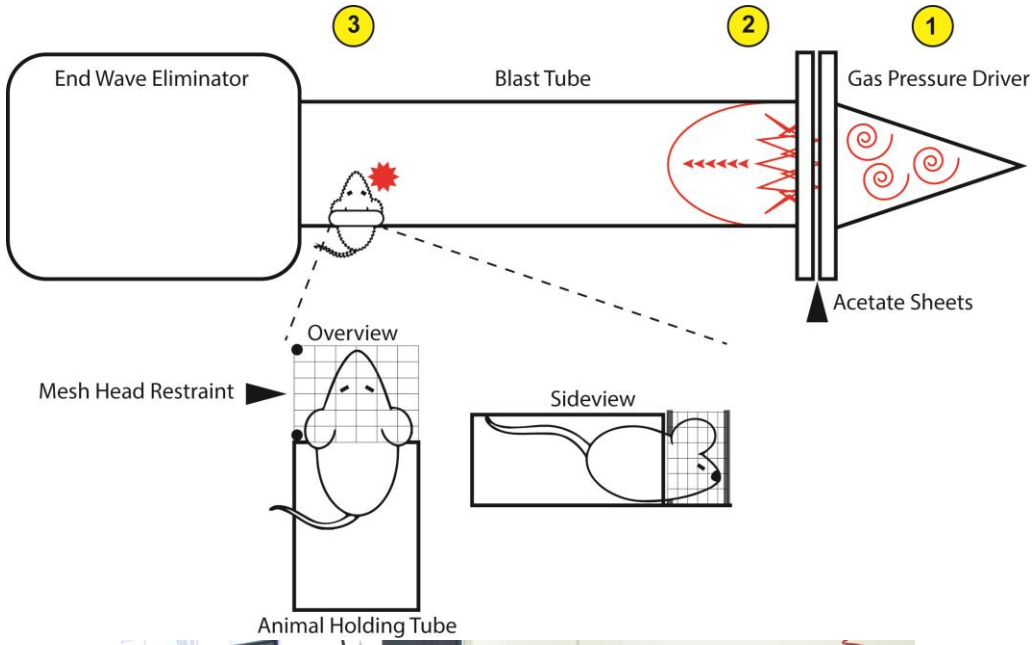
4.1. Advanced Blast Simulation and Plasma Collection

Blast exposure procedure will be discussed in published work in section 5 below. Briefly, anesthetized adult, male Sprague-Dawley (S-D) rats are anesthetized with 3% isoflurane in 100% oxygen before being securely placed within an animal holding tube. The S-D rats are positioned within the tube in such a way to allow head-only lateral exposure to the blast along the length of the blast tube. The head of the animal is securely restrained atop a small platform by mesh netting (restraint), or not restrained (whiplash), or is covered by a protective netting material (net). Helium gas pressure is built up within the pressure driver section and, depending on the thickness of the plastic acetate sheets barrier, will produce a single primary blast overpressure wave of 15-30 pounds per square inch (PSI) (103.4-206.8 kilopascals (kPa)) that bursts through the divider and travels along the blast tube to impact the anesthetized animal along the side of the head. Reflective blast waves are prevented by the end wave eliminator device at the distal end of the blast tube to ensure single blast pulse exposure only. Anesthetized sham blast exposure trials are also performed. Following blast, still unconscious animals are removed from their holding tube and allowed to recover consciousness before being returned to the animal holding facility. Following 24 hours after blast or sham experiments, animals are decapitated and exsanguinated. Small blood samples are collected via potassium ethylene diamine tetra-acetic acid coated tubes (BD Vacutainer K_2EDTA #367841) and then centrifuged for 10 minutes at 2000 g. Plasma separated fraction is aliquoted within 1.5 mL Eppendorf tubes to be stored at -80˚C until use. Blast exposure experiments and blood sample collection was performed under Dr. Thomas Sawyer and coworkers at the Defence Research and Development

Canada (DRDC) location in Suffield, AB, Canada. **See figure 4.1** for schematic overview of blast exposure procedure.

Figure 4.1. Advanced Blast Simulation device. (A) 1) Built up helium gas pressure within the driver section **2)** ruptures the cellulose acetate diaphragm to produce a single overpressure wave that travels down the length of the blast tube to **3)** impact the anesthetized S-D rat along the side of the head only. **(B)** Actual photograph of blast tube at DRDC Suffield (reprinted with permission).

A



B

4.2. Immunohistochemistry

At one day after blast exposure, animals were euthanized via decapitation and immediately perfused transcardially with ice-cold phosphate-buffer saline (PBS; 0.1 M, pH 7.4) followed by 4% formaldehyde (BDH #0500-4LP) in PBS fixative solution. The brains were then removed from the calvarium and post-fixed with 4% formaldehyde in PBS at 4˚C overnight (O/N) before histological preparations. To prevent ice crystal formation in fixed tissue sections, brains were dehydrated in 30% sucrose in PBS cryoprotectant solution at 4˚C until brains sank to the bottom of their tubes. Brains were then immersed and bathed in Tissue-tek optimal cutting temperature (Tissue-Tek OCT) solution and placed within a tissue mold (Tissue-tek Cryomold). The mold is filled with OCT solution and then flash frozen with liquid nitrogen before storing at -80˚C until use.

Coronal sections (30 μM thickness) of rat brain containing the hippocampus ipsilateral to blast exposure were prepared within a cryostat (Shandon Cryotome) and post-fixed as free floating sections. Tissue sections are rehydrated in PBS at room temperature (RT) for 10 minutes before permeabilization in 0.1% Triton X100 in PBS for another 10 minutes at RT. Sections were blocked in 2% bovine serum albumin (BSA) in PBS for 45 minutes at RT. Sections were gently washed in 0.5% BSA in PBS (PBB). Sections were sequentially immunostained with primary antibodies and fluorescence tagged secondary antibodies against either phosphorylated NFH (pNFH, Covance SMI 31, 1:500, Cedarlane, Burlington, ON, Canada) or non-phosphorylated NFH (NFH, Convance SMI 32, 1:500, Cedarlane) and GFAP (Invitrogen 18-0063, 1:1000, Burlington, ON, Canada). Primary antibody are diluted in PBB and added to tissue sections for incubation for 60 minutes at RT or O/N at 4˚C. Free floating sections are rinsed five times in PBB. Green fluorescent-dye tagged secondary antibody for

pNFH or NFH (Alexa Fluor 488 Z25002, Invitrogen, 1:1000) and red dye tagged antibody for GFAP (Alexa Fluor 594 Z25307, Invitrogen, 1:1000) is added to the sections for 60 minutes at RT. Sections are then rinsed five times in PBB and an additional five times in PBS. Sections are then counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 300nM, Invitrogen D1306) in PBS for five minutes to view nuclei (blue). Sections are washed three times in PBS before sections are mounted onto microscope slides with a coverslip mounted on topusing gelvatol in tris-[hydroxymethyl] aminomethane (Tris)-Cl solution (0.2 M, pH 8.5).

Stained brain slices were viewed with a Quorum WaveFX laser scanning confocal microscope and images were captured with a Hamamatsu EM-CCD camera (Quorum Technologies WaveFX-X1, Guelph, Ontario). Finally, captured images were stitched together using the software package MetaMorph (Molecular Devices) to show structures of different brain regions. Areas of interest were then cropped to include approximately 500 µm sections for presentation.

4.3. Sports Concussion Pilot Project

4.3.1. Athletes and non-athletes recruitment

The study was approved by the Biomedical Research Ethics Committee of the University of Saskatchewan, Canada (Bio # 13-195). Members of University of Saskatchewan Huskies Athletic teams including Canadian football, ice hockey, basketball, and soccer teams as well as healthy non-athlete male and female university students were asked to participate in the investigation. Individuals were asked in a questionnaire whether they were in good standing health without any existing illnesses or condition and whether they had recently (<6 months) suffered a head injury. Those who had a pre-existing condition or had suffered a recent injury

were excluded from the study to prevent potential confounds. Altogether, participants of high-contact sports were recruited as follows: ice hockey (n=17), football (n=20), soccer (n=4), basketball (n=18), and wrestling (n=6). Samples were also collected from athletes in typically low contact sports such as volleyball and cross country (n=11). For normal values, 27 additional samples were collected from the non-athlete university student population. In total, 103 participants submitted a blood sample. For summary of participant characteristics see table 6.1 in section 6. Initial samples submitted by athletes before pre-season training are treated as baselines, and should they suffer a significant head injury throughout the season a subsequent post-TBI sample is collected. In total six concussed athletes were identified during the season using the sports concussion assessment tool (SCAT)3 concussion assessment criteria (Guskiewicz, et al. 2013) and their post-concussion blood samples collected 1-7 day post-incidence depending on subjects availability.

4.3.2. Plasma Separation & Storage

All sample collection was performed following signed and informed consent prior to invasive procedure and sample testing as outlined by the Tri-Council Policy Statement (TCPS)2. Samples were alphanumerically coded for subsequent single-blinded testing. A small sample of venous blood (2 mL) is collected from individuals into lithium heparin coated vacutainer tubes (BD vacutainer PST #367962). Samples were immediately placed on ice to allow stable transport to laboratory setting. Samples were then centrifuged at 10,000RPM for 10 minutes for plasma isolation. Plasma fraction was aliquoted in 1.5 mL Eppendorf tubes and immediately stored at -80˚C for future analysis.

4.4. Plasma PrP^C ELISA

For sensitive quantification of full-length soluble PrP^C, we employed an ELISA technique using a commercially available qualitative assay kit (Spi Bio A05201, Paris, FR) and modified the manufacturer's protocol to allow sensitive and accurate quantification. Pure full-length recombinant PrP^C (Prionatis, α-Rec Mouse PrP-RPA0101S, Zurich, CH) was used for producing serial dilutions (0.625—20 ng/mL) in order to establish the calibration curve for quantifying samples (**see figure 4.2**). All samples and PrP^C protein standards were diluted in the manufacturer's provided dilution buffer solution (1 M phosphate, 1% BSA, 4 M NaCl, 10mM EDTA, and 0.1% sodium azide). Remaining solutions and lyophilized reagents provided by the manufacturer were reconstituted and prepared according to the suggested protocol. Briefly, overall protein concentration of individual samples was first determined in triplicate using the Bio-Rad DC protein assay (Bio-Rad #500-0113/0114). Samples and standards were loaded in equal volume in triplicate in pre-coated 96-well microplate strips. Diluted samples were loaded as such that each well contained approximately overall protein amounts of 75-100 µg. The plate was then incubated O/N at 4°C with shaking to allow adequate antigen binding to well-embedded monoclonal antibodies, specific to the 144-153 amino acid residues within the C-terminus. After rigorous washing (4M phosphate, pH 7.4) to remove unbound particles, the wells are incubated with an acetylcholinesterase- (AChE) Fab' conjugated antibody solution targeting the N-terminal OR between residues 51-90 for two hours at RT with shaking to form a "sandwich complex" of solid phase antibody—PrP^C—enzyme-conjugated antibody. Unbound antibody is washed away after another cycle of washing before adding modified Ellman's reagent with substrate (5,5'-dithio-bis(2-nitrobenzoic acid) or DTNB; 0.1 M phosphate buffer, 0.5 mM DTNB, 1 mM acetylthiocholine iodide, pH 7.4). The assay uses thiol ester

acetylthiocholine iodide, which undergoes hydrolysis via AChE to produce thiocholine and acetate. This thiol group then reduces DTNB ion resulting in cleavage of the disulfide bond to produce 5-thio-2-nitrobenzoate (TNB^-), which ionizes at neutral and alkaline pH to form TNB^{2-}. TNB^{2-} is responsible for producing the yellow color in solution proportional to the amount of captured protein (**see Figure 4.3** for reaction diagram). This colorimetric reaction and can be quantified in a spectrophotometer (SpectraMax M5, Molecular Devices) by measuring absorbance of visible light at 405nm. Raw absorbance values were obtained in software platform Softmax 6.4 (Molecular Devices) and interpolated along the standard calibration curve for conversion into PrP^C concentration values.

Figure 4.2. PrP^C ELISA calibration curve. Representative plot of pure recombinant human PrP^C serial standards (0.625—20ng/mL) assayed in triplicate using a modified commercial ELISA kit. Optical density values read at 405nm wavelength were obtained and plotted using Microsoft Excel. Linear regression equation y=0.0514x – 0.0026 where y=OD and x=concentration is used to interpolate the latter when absorbance values (y) are obtained.

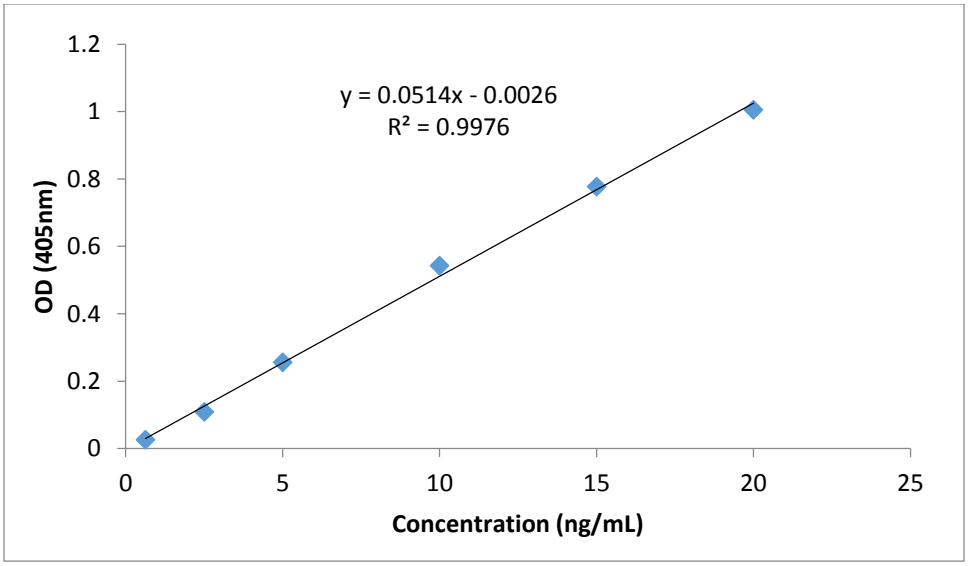
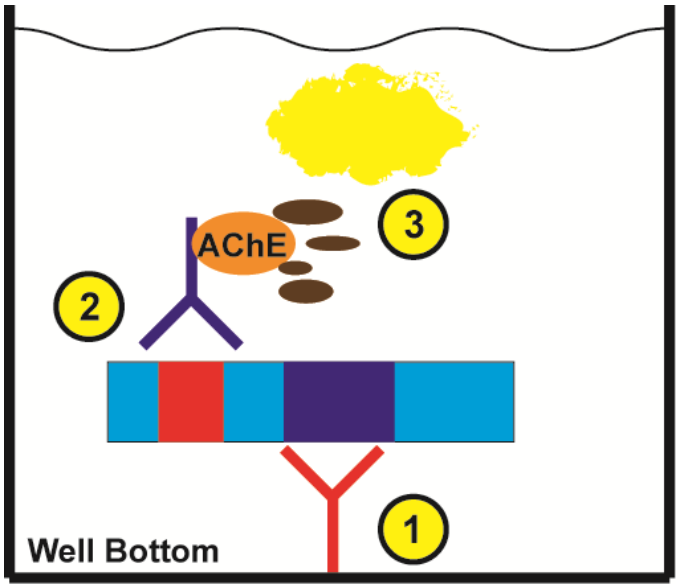


Figure 4.3. PrP^C ELISA Diagram. (1) Well-embedded monoclonal antibody binds to the 144-153 residue segment of PrP^C. (2) AChE-conjugated monoclonal antibody binds to the N-terminal OR at residues 51-90. (3) Ellman's reagent with substrate is added to produce yellow color in solution proportional to the amount of bound AChE-conjugated antibodies, and thus captured PrP^C.



4.5. Western Blotting

Western blotting was performed as previously described (Pham, et al. 2014). Protein concentration of plasma samples was determined using the Bio-Rad DC assay and 30µg protein per well was loaded into 15% acrylamide gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein was then transferred onto polyvinylidene fluoride (PVDF) membrane (FluoroTrans, Pall Life Sciences) at 100V for 1 hour. Membranes were blocked in 5% BSA in PBS-Tween 20 (0.1%) at RT for 1 hour. Primary antibodies used for immunoblotting are as follows: PrP^C (Santa Cruz sc-7693, 1:500), GFAP (Santa Cruz sc-6170,

1:500), ADAM10 (Abcam ab84595, 1:1000), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam ab9484, 1:1000), and actin (Santa Cruz sc-1616, 1:500). Primary antibody incubation was performed either at RT for 1-2 hours or at 4°C O/N. Following stringent washing and secondary antibody incubation steps, membranes were exposed to enhanced chemiluminescence (ECL) reagent (Amersham #45000875) and exposed to X-ray film. Protein bands of interest were analyzed using NIH ImageJ software and normalized to that of the actin loading control in each sample lane.

4.6. Statistical Analysis

Statistical analysis for all data was performed using software platforms IBM Statistical Package for the Social Sciences (SPSS) 21 and Graphpad Prism 5. All non-parametric statistical methods for analyses of plasma PrP^C in blast-exposed rats is discussed below in section 5.1. Immunohistochemical comparison of relative fluorescence of targeted proteins in stained hippocampal sections was performed using student's *T*-test (see section 5.2). Comparison of plasma PrP^C concentration in restraint, whiplash, and net groups was performed using one-way analysis of variance (ANOVA) of results distribution, and post-hoc Dunnett's multiple comparison test for significant pairings of blast groups and control (see section 5.1 and 5.2). Immunoblotting comparison of target proteins was also analyzed using one-way ANOVA and post-hoc Dunnett's multiple comparison test for blast groups against control (see section 5.3). For sports concussion pilot experiments, *T*-test was performed for plasma PrP^C mean value comparison of the following pairings: male vs. female, athletes vs. non-athletes, and post-TBI vs. baseline or combined athletes and non-athletes (see section 6.1). One-way ANOVA was used to determine whether there is significant variation of mean PrP^C concentration among different age

groups. Immunoblotting comparison of target proteins in human samples was performed using *T*-test (see section 6.2). For calculation of the reference interval (RI) of children's plasma PrP^C concentration as well as corresponding 90% CIs, non-parametric methods were used to establish the 95th percentile (see section 6.3). *T*-test was performed for comparison between mean concentration values between genders. Scatterplot and linear regression analysis was performed for trend determination between PrP^C concentration and age progression. Results were considered statistically significant when $p \leq 0.05$.

Table 4.1. Chemical and biological reagents, equipment and supplies

Materials, Chemicals, and Biological Reagents	**Supplier**
Actin I19 sc1616 goat polyclonal antibody	Santa Cruz
ADAM10 ab84595 rabbit polyclonal antibody	Abcam
Alexa Fluor 488 Z25002 green mouse IgG1 labelling dye	Life Technologies
Alexa Fluor 594 Z25307 red rabbit IgG labelling dye	Life Technologies
Bovine Serum Albumin	Sigma Aldrich
Cryotome Shandon	Life Sciences
DAPI D1306 nuclear dye	Life Technologies
DC Protein assay #500-0113/-0114	Bio-Rad
ECL Amersham #45000875	VWR
Formaldehyde BDH0500-4LP	VWR
GAPDH ab9484 mouse monoclonal antibody	Abcam
Gelvatol (polyvinyl alcohol) #341584	Sigma Aldrich
GFAP #18-0063 rabbit polyclonal antibody	Life Technologies

GFAP C19 sc-6170 goat polyclonal antibody	Santa Cruz
Tissue-Tek Optimal Cutting Temperature (OCT) Compound	VWR
Tissue-Tek Cyromold	VWR
NFH SMI 32 mouse monoclonal antibody	Cedarlane
p-NFH SMI 31 mouse monoclonal antibody	Cedarlane
PBS	Produced in lab
PrP^C C20 sc-7693 goat polyclonal antibody	Santa Cruz
PrP^C ELISA A05201	Cedarlane
PrP^C recombinant mouse protein PrP-RPA0101S	Prionatis
PVDF Fluor Trans	VWR
Quorum WaveFX-X1 confocal microscope	Quorum Technologies
Spectramax M5 spectrophotometer Molecular Devices	VWR
Tris-Cl	Produced in lab
Triton X100 T8787	Sigma Aldrich
Vacutainer K_2EDTA #367841	Fisherbrand
Vacutainer PST #367962	Fisherbrand

Section 5. Blast Exposure Results

5.1. Primary Blast-Induced Traumatic Brain Injury in Rats Leads to Increased Prion Protein in Plasma: A Potential Biomarker for Blast-Induced Traumatic Brain Injury

Nam Pham,[1] Thomas W. Sawyer,[2] Yushan Wang,[2] Ferdous Rastgar Jazii,[1] Cory Vair,[2] and Changiz Taghibiglou[1]

Abstract

Traumatic brain injury (TBI) is deemed the signature injury of recent military conflicts in Afghanistan and Iraq, largely because of increased blast exposure. Injuries to the brain can often be misdiagnosed, leading to further complications in the future. Therefore, the use of protein biomarkers for the screening and diagnosis of TBI is urgently needed. In the present study, we have investigated the plasma levels of soluble cellular prion protein (PrPC) as a novel biomarker for the diagnosis of primary blast-induced TBI (bTBI). We hypothesize that the primary blast wave can disrupt the brain and dislodge extracellular localized PrPC, leading to a rise in concentration within the systemic circulation. Adult male Sprague Dawley rats were exposed to single pulse shockwave overpressures of varying intensities (15-30 psi or 103.4 206.8 kPa] using an advanced blast simulator. Blood plasma was collected 24 h after insult, and PrPC concentration was determined with a modified commercial enzyme-linked immunosorbent assay (ELISA) specific for PrPC. We provide the first report that mean PrPC concentration in primary blast exposed rats (3.97 ng/mL – 0.13 SE) is significantly increased compared with controls (2.46 ng/mL – 0.14 SE; two tailed test $p < 0.0001$). Furthermore, we report a mild positive rank correlation between PrPC concentration and increasing blast intensity (psi) reflecting a plateaued response at higher pressure magnitudes, which may have implications for all military service members exposed to blast events. In conclusion, it appears that plasma levels of PrPC may be a novel biomarker for the detection of primary bTBI.

Key words: blast exposure; blood plasma; brain injury; ELISA; PrPC

Introduction

TRAUMATIC BRAIN INJURY (TBI) is the leading cause of mortality and disability in individuals under < 45 years of age in North America, and is estimated to occur in 600/100,000 people.[1 3] Most cases of TBI are a result of a significant impact or penetrating injury to the head leading to the disruption of normal brain functions.[4] The leading causes of TBI include vehicle accidents, falls, assaults, and sports-related injuries. However, over the past decade, the scope of concern over TBI has expanded to include injuries sustained during military operations and urban terrorist activities.[5] Since the beginning of the global war on terror, it is estimated that 15 20% of returning service members have sustained a TBI, representing > 300,000 cases for the period between 2001 and 2014.[6,7] Therefore, TBI is described as the signature injury of these con icts and has been associated with a spectrum of post-deployment health issues, including decline in long-term motor, psychosocial, and cognitive ability; sleep disturbance; psychiatric disorders; post-concussive syndrome; substance abuse; and chronic traumatic encephalopathy.[8 12] Of all military-related TBI incidences, blast exposure is by far the leading cause, accounting for ~47% of TBI cases in Afghanistan and 64% of those in Iraq, thereupon establishing blast-induced TBI (bTBI) as its own category distinct from the spectrum of non-blast TBI.[13,14]

The rising incidence of bTBI in the military over the past decade has been largely the result of the expanded use of various explosive munitions such as grenades, improvised explosive devices (IEDs), and land mines.[9,15] Moreover, improvements in medical treatment and protective equipment have increased the survival rate, concomitant with increased TBI reporting in those sustaining injuries previously considered fatal.[16,17] Despite these advancements, bTBI remains a large concern, as the effects of blast exposure, particularly chronic exposure, to the human brain are not clearly understood because of their complex and heterogeneous nature.[18] During

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an explosive detonation, the instantaneous conversion of a solid material into gas produces a supersonic overpressure wave, termed the primary blast wave.[10] Additional byproducts of this reaction can include extreme heat, toxic gases, electromagnetic pulses, and winds generated by the abrupt air pressure changes produced in the blast wave.[10,13,19–21] Considering that the manifestations of bTBI may be a consequence of certain or even all such blast-induced forces, there is no clinical standard for assessing bTBI other than using conventional TBI diagnostic guidelines. Of the various patterns of bTBI, those caused by the primary blast wave itself are the least recognized and understood.[22] Therefore, considerable effort has been directed toward the development of reliable screening procedures to help determine the degree of head trauma suffered by military service personnel.

To date, various clinical assessment guidelines and advanced neuroimaging techniques remain the standard tools for determining bTBI; however, there is no guarantee of proper diagnosis and timely management, because of the inconsistent indications of bTBI. A promising approach to circumvent these uncertainties has focused on the detection of protein biomarkers symptomatic of bTBI. Typical markers examined for bTBI may include such proteins as S100 calcium binding protein B (S100B), glial brillary acidic protein (GFAP), neuron speci c enolase (NSE), cleaved tau (C-tau), and various others.[23–27] However, to our knowledge, there are no reports indicating cellular prion protein (PrPC) as a potential biomarker of bTBI. The present study seeks to address this question as to whether assessment of plasma PrPC may also be indicative of bTBI.

PrPC is a ubiquitous glycoprotein distributed throughout many cell types and tissues in mammals, with preponderance within the central nervous system (CNS).[28–30] PrPC is 208–209 amino acids long, and is almost entirely located extracellularly on dynamic lipid raft domains tethered by a glycophosphatidylinositol (GPI) anchor.[30,31] Efforts to elucidate the physiological role of PrPC in the CNS have determined its involvement in various functions, including cellular adhesion, cell signaling, ion homeostasis, and neuroprotection.[32–44] Because of PrPC s extracellular nature, it is possible that during blast exposure, shearing forces from the primary blast wave traversing across the brain may cause the tenuously bound PrPC to dislodge and collect within the systemic circulation. We addressed this hypothesis by subjecting adult male Sprague–Dawley (S-D) rats to controlled single pulse shockwaves closely simulating free- eld blast and collecting blood plasma afterwards for quanti - cation of PrPC.[45] In this study, we established 1) plasma PrPC as a potential biomarker for primary bTBI and 2) a positive correlation between plasma PrPC and blast wave intensity exposure.

Methods

Advanced blast simulator (ABS)

A custom-built ABS (~30.5cm in diameter and 5.79 m in length) located at Defence Research and Development Canada (DRDC) Suf eld was used for producing simulated blast waves.[45] The ABS consisted of a driver section lled with high-pressure gas, and a low-pressure test section, separated by a frangible cellulose acetate diaphragm. Closely controlled pressurization of the driver causes rupture of the diaphragm, releasing high-pressure gas into the test section, and generating a shockwave down the length of the test section. The inclusion of a custom-designed divergent driver and an end wave eliminator in this ABS system enables the highly reproducible generation of single pulse shockwaves.[45] Compressed helium and varying thicknesses of cellulose acetate sheets were employed to obtain the desired target pressure. A complete description of the development and operation of the ABS is described in a manuscript nearing completion, to be submitted to the open literature by Sawyer and colleagues.

Animal exposure to simulated blast

In conducting this research, the authors adhered to the *Guide to the Care and Use of Experimental Animals* and *The Ethics of Animal Experimentation*, published by the Canadian Council on Animal Care. Adult male S-D rats were acquired from Charles River Laboratories (St. Constant, Quebec, Canada) and acclimated for at least 1 week prior to exposure. The animals were kept on a 12 h light/dark cycle and fed ad libitum. On the day of use, the animals (~350–400 g) were anaesthetized with 3% iso urane in oxygen for 3 min in a closed induction chamber. Anesthesia was maintained using a face mask, and the animal was placed into a restraint consisting of a clear plastic cylindrical sleeve, with the neck encircled in a closely- tting plastic collar with the head protruding from the end. The hindquarters were supported using an end cap- tted with a piston. To the left of the head, a mesh netting was secured between two pins placed vertically in line with the side of, and above and below the head. The head was placed against this vertical netting, and then held in place using additional netting around the head. This was locked into place using Velcro on the side of the head opposite the direction of shockwave propagation. After a minimum of 8 min of anesthetic exposure, the cylindrical restraint containing the animal was set into the wall of the ABS, such that only the head protruded into the test section. Test groups consisted of sham control, and head-only, side-on exposures of single pulse shockwave overpressures of 15, 20, 25, and 30 psi or 103.4, 137.9, 172.4, or 206.8 kPa.

After exposure, the animals were immediately removed from the shock tube and animal restraint, and were closely observed for at least 30 min post-exposure, or until no signs of stress were evident. The animals were returned to the dedicated animal holding facility where they had been observed on a daily basis prior to testing. At 24 h, the animals were anaesthetized and euthanized by decapitation prior to blood sample collection.

Sample collection

Following anesthesia, whole trunk blood samples were collected from both control ($n = 19$) and blast ($n = 33$) group rats following decapitation into potassium ethylene diamine tetraacetic acid (K_2EDTA) coated blood collection tubes. Samples were centrifuged for 10 min at 2000 *g*, and the separated plasma supernatant was collected. To avoid excessive freeze–thaw cycles, blood plasma aliquots were made and stored at −20°C for short-term use, and the rest were stored at −80°C.

Plasma PrPC ELISA

For sensitive quanti cation of full-length soluble PrPC, we employed an ELISA technique using a commercially available assay kit (Spi Bio A05201, Paris, France). The kit is typically used for qualitative screening in animal products; therefore, we modi ed the manufacturer s protocol to allow sensitive and accurate quanti cation. Pure full-length recombinant PrPC (Prionatis, α-Rec Mouse PrP-RPA0101S, Zurich, Switzerland) was used for producing serial dilutions (0.625–20 ng/mL) in order to establish the calibration curve for quantifying samples. All samples and PrPC protein standards were diluted in the manufacturer s provided dilution buffer solution (1 M phosphate, 1% bovine serum albumin [BSA], 4 M NaCl, 10 mM EDTA, and 0.1% sodium azide). Remaining solutions and reagents provided by the manufacturer were reconstituted and prepared according to the suggested protocol.

Brie y, overall protein concentration of individual samples was rst determined in triplicate using the Bio-Rad DC protein assay (Sigma-Aldrich, bovine albumin, A-9647, Oakville, ON). Samples

and standards were loaded in equal volume in triplicate in the kit s 96 microwell plate strips. Diluted samples were loaded such that each well contained overall protein amounts of ~ 75 100 μg. The plate is then incubated overnight at 4°C with shaking to allow adequate antigen binding to well-embedded monoclonal antibodies, speci c to the 144 153 amino acid sequence. After rigorous washing (4M phosphate, pH 7.4), the wells were incubated with an acetylcholinesterase- (AChE) Fab conjugated antibody solution for 2 h at room temperature (RT) with shaking, thus completing a double-antibody sandwich. After another cycle of rigorous washing, Ellman s reagent was added in equal volume to each well, and incubated in the dark for 30 min at RT with shaking. Any immobilized AChE-conjugated antibody bound to PrPC therefore reacted with Ellman s reagent to produce a colorimetric reaction in solution proportional to the concentration of PrPC, which was read using a microplate reader at 405 nm (Molecular Devices, LLC., SpectraMax M5, Sunnyvale CA). Raw absorbance values were interpolated along the standard calibration curve and converted into PrPC concentration values.

PrPC Western blotting

Western blotting was conducted as previously described.[46] Brie y, diluted plasma samples were separated with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred onto a polyvinylidene di uoride (PVDF) membrane, and probed with an anti-PrPC primary antibody (Santa Cruz, goat IgG anti-PrP C-20 pAb, 1:500, sc-7693). For sequential reprobing of the same blots, the membranes were stripped and subjected to immunoblotting with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody (AbCam, mouse IgG2b mAb, 1:2000, ab9484). Blots were developed using enhanced chemiluminescence detection (Amersham) and exposed to x-ray lm. Band intensities were quanti ed using National Institutes of Health (NIH) ImageJ software and normalized to the quantity of GAPDH in each sample lane. Blots were developed in triplicate, and a representative image is provided (see Fig. 1).

Statistical analysis

Statistical analysis for all data was performed using the IBM SPSS 21 Statistical Package. Nonparametric data were appropriately analyzed using the Kruskall Wallis test for comparison of mean rank values of control and the different blast intensity groups. Post-hoc Mann Whitney U test with a Bonferroni correction for 95% con dence interval (CI) was used for determining statistical signi cance between mean rank values of control and individual blast group PrPC concentration. The Jonckheere trend test was used to determine a signi cant relationship between blast intensity and PrPC concentration. Kendall s tau-b test determined the nature and degree of association for said relationship. Receiver operating characteristic (ROC) analysis was performed for determining accuracy of classi er performance. The measure of general predictiveness of classi ers was determined by area under the ROC curve (AUC). Two-graph ROC (TG-ROC) analysis was used for determining the cutoff value, as described by Greiner and coworkers, between control and blast exposure groups, and positive and negative predictive values (PPV and NPV) were subsequently calculated.[47] For all tests, statistical signi cance was determined when $p \leq 0.05$.

Results

The use of the ABS system, in concert with the head restraint con guration employed in this work, has been shown to minimize concussive and whiplash forces and produce a clean primary single pulse shockwave insult (manuscript in preparation by Sawyer et al.). Figure 2 shows a representative example of the single pulse shock wave produced with a 25 psi target pressure. The overpressures obtained for the four test groups were: 15 – 0.2, 20 – 0.8, 25 – 0.3, and 30 – 0.9 psi (mean – SD). Immediately after exposure, the animals showed no obvious signs of injury and revived from the anesthetic with no visible differences compared with sham controls with respect to time to revival and time to mobility. No signs of distress or injury were noted either immediately after regaining consciousness after exposure, or the following day.

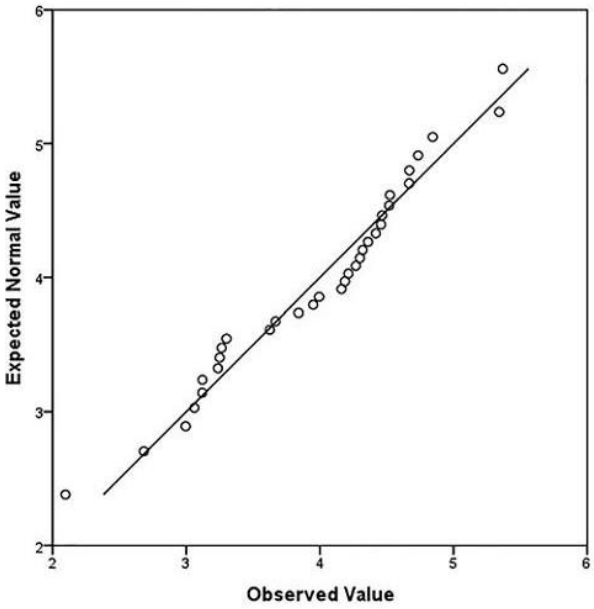

FIG. 1. Quantile-quantile Q-Q plot of blast soluble cellular prion protein (PrPC) distribution. Most of the blast group PrPC concentration results depart from the normal distribution reference line ($y = x$). Therefore, data are not normally distributed and are considered nonparametric.

Quantitative analysis of blood plasma PrPC from both control ($n = 19$, 0 psi) and blast ($n = 33$, 15 30 psi) groups was performed using a modi ed commercial ELISA kit speci c for PrPC; for results summary see Table 1. Graphic representation of PrPC concentration results is provided in a box-and-whisker plot (see Fig. 3) showing that the majority of blast group values lie above the control group median value (2.66 ng/mL), indicating that blast group concentrations are distinct from control results. A quantile-quantile (Q-Q) plot of blast group results distribution reveals that most data points deviate from the normal distribution line ($y = x$), and, therefore, these data are considered nonparametric (see Fig. 1). As such, the Kruskall Wallis test for nonparametrics was appropriately used for determining differences in PrPC concentration mean rank values of sham controls and individual blast intensity groups. There was a statistically signi cant difference ($\chi^2 = 31.62$, $p < 0.0001$) between sham control (mean rank = 11.84, $n = 19$), 15 psi (mean rank = 31.14, $n = 7$), 20 psi (mean rank = 40.00, $n = 7$), 25 psi (mean rank = 37.58, $n = 12$), and 30 psi (mean rank = 29.14, $n = 7$) blast exposure groups. Post-hoc Mann Whitney U test with a Bonferroni correction for multiple comparisons with an adjusted level of signi cance ($\alpha = 0.0125$) determined statistical difference of PrPC concentration mean rank between sham controls and 15 psi (10.89 vs. 20.57, $U = 17$, $p = 0.004$), 20 psi (10.05 vs. 22.86, $U = 1$, $p = 0.0001$), 25 psi (10.16 vs. 25.25, $U = 3$, $p < 0.0001$), and 30 psi (10.74 vs. 21.00, $U = 14$, $p = 0.002$) blast exposure groups.

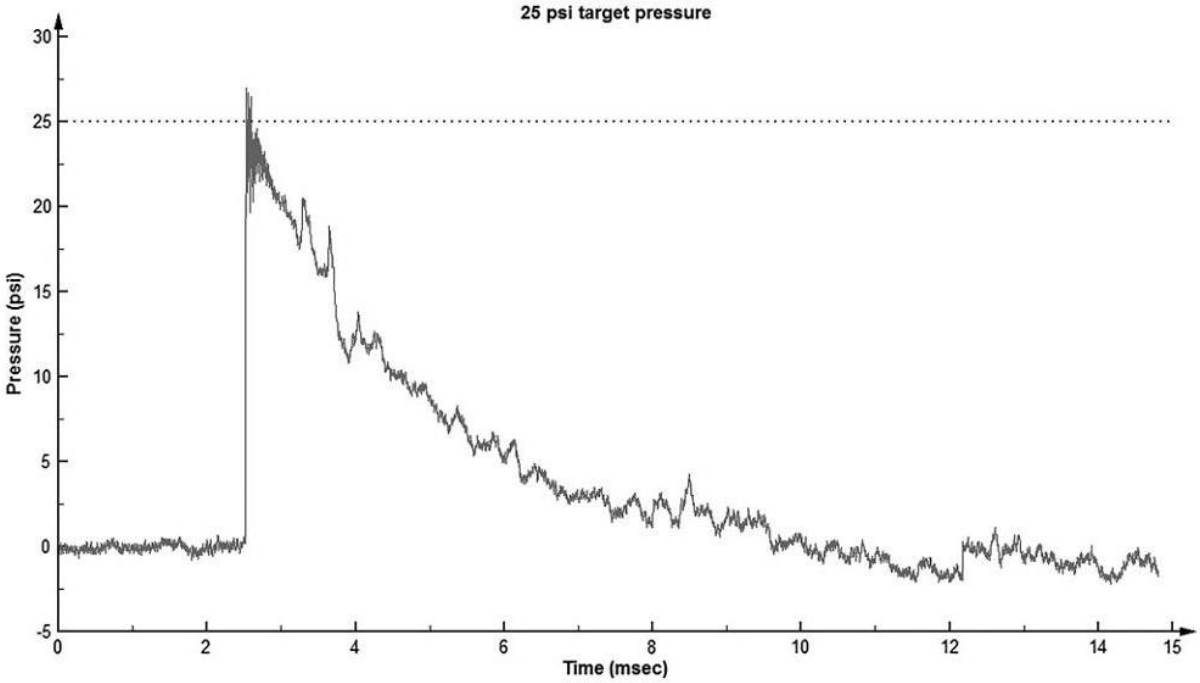

FIG. 2. Primary blast profile: Representative single pulse wave form generated in the advanced blast simulator (ABS) at the test location at a target overpressure of 25 psi. The pressure perturbations at ~ 3.6 4.0 msec are caused by the placement of the head within the shock ow.

Quantified differences between blast and control group PrPC concentration is demonstrated with Western blotting (see Fig. 4). Densitometric analysis using NIH ImageJ software calculated PrPC band intensity in relation to GAPDH loading control in blast group plasma determined a 1.60 – 0.41 fold increase ($n = 4$, two tailed test $p < 0.001$) when compared with controls. To determine a signi cant relationship between increasing blast pressure intensity (psi) and plasma PrPC content, Jonckheere trend test was used, which showed an ordered relationship between blast intensity and PrPC concentration ($J\text{-}T = 773.00$, $p < 0.0001$). Additionally, Kendall s tau-b test determined the correlation coef cient at 0.446 ($p < 0.0001$), re- ecting a positive trend association between increasing blast intensity groups and their respective median PrPC concentrations.

ROC analysis was performed for determining accuracy of our ELISA test based on the predictability of control and blast group classi ers (see Fig. 5). ROC analysis allows comparison of PrPC sensitivity against the inverse speci city over a range of thresholds for evaluating overall test accuracy. The AUC was determined at 0.944 – 0.032 SE (95% CI, 0.881 1.000, $p < 0.0001$) indicating ELISA test results to be highly accurate for distinguishing between control and blast groups. As there is presently no standard reference database available for rat plasma PrPC, we performed TG-ROC analysis using values obtained to determine the minimum cutoff value de ning blast exposure. We chose a conservative cutoff of 2.78 ng/mL, which yielded 79.1% sensitivity and speci city, 81.6% PPV, and 85.7% NPV.

TABLE 1. PLASMA PRPC ELISA RESULTS SUMMARY

Group	*Target pressure (psi)*	*Actual pressure (psi)*	n	*PrPC Concentration (ng/mL)*		
				Mean – SE	*Median*	*Range*
Sham control	0	0	19	2.46 – 0.14	2.66	0.67 3.35
Blast	15	15 – 0.2	7	3.74 – 0.34	3.99	2.10 4.67
	20	20 – 0.8	7	4.27 – 0.26	4.47	3.27 5.34
	25	25 – 0.3	12	4.18 – 0.18	4.26	3.06 5.37
	30	30 – 0.9	7	3.54 – 0.30	3.25	2.68 4.84
	15-30		33	3.97 – 0.13	4.19	2.10 5.37

Blood plasma from control ($n = 19$, 0 psi) and blast ($n = 33$, 15 30 psi) group rats were assayed using a modi ed commercial PrPC ELISA kit for quantification. Individual results not provided.

PrPC, soluble cellular prion protein; ELISA, enzyme-linked immunosorbent assay.

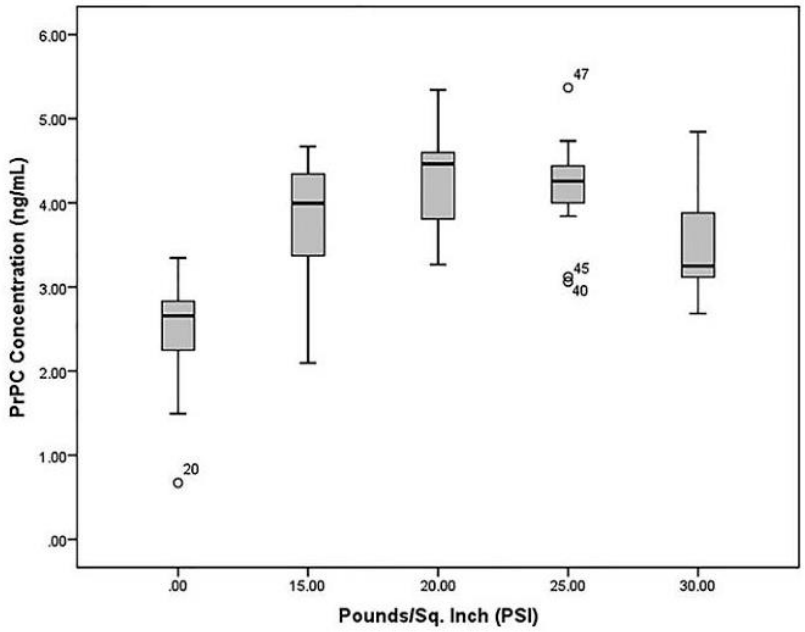

FIG. 3. Box-and-whisker plot of soluble cellular prion protein (PrPC) concentrations. Box plot comparison of control (0 psi, $n=19$) and blast (15 psi, $n=7$; 20 psi, $n=7$; 25 psi, $n=12$; 30 psi, $n=7$) groups illustrate that the majority of blast group PrPC concentrations (interquartile range Q1-Q3) lie above the median (Q2) of the control group. Data points 20, 40, 45, and 47 are considered outliers from group distribution.

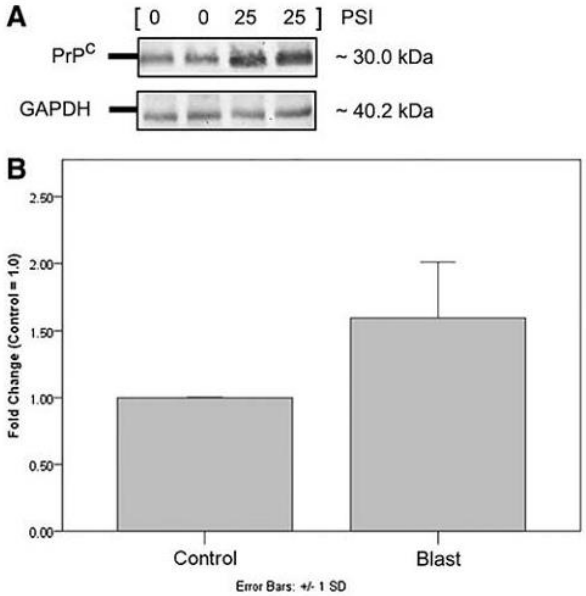

FIG. 4. Western blot of soluble cellular prion protein (PrPC) **(A)** Results are semiquantitative, and are for the purpose of simple visualization of increased PrPC in blast group plasma compared with control. **(B)** Numerical (fold) change bar graph represents a mean fold increase of 1.60 – 0.41 compared with control values, given an arbitrary value of 1.0 ($n=4$, two tailed test $p<0.05$).

Discussion

Increased blast exposure during the recent military con icts in Afghanistan and Iraq has not surprisingly been concomitant with increased reports of TBI among service members.[6,16,17] TBI is typically brought about by direct impact or acceleration forces to the head, leading to collision between the brain and skull, as well as shearing strain on brain tissue and vasculature.[3,48] Proper diagnosis of TBI caused by blast is especially dif cult, given the potential absence of physical symptoms or presence of nonspeci c ones, thus confounding the recognition of mild indications such as sleep disturbance, fatigue, headaches, and loss of concentration that are often overlooked and underreported by service members.[9,49] A possible approach toward addressing this issue is in screening individuals for protein biomarkers speci c for bTBI. Various proteins have been investigated, but none has been conclusively established as having clinically practical screening qualities.[23,24] For example, the S100B protein is frequently used as a biomarker for TBI, and has been thoroughly investigated because of its strong NPV; however, its value for predicting TBI outcome is questionable, because of its high correlation with bone fractures without TBI, extracranial injury, and even melanoma.[50–53] Another protein, GFAP, has shown correlation with TBI outcome, but has been inconsistent in discerning between those with TBI and uninjured victims.[54] As such, investigation toward establishing both a highly predictive and reliable protein biomarker continues. Therefore, in collaboration with the DRDC, Suf eld Research Center, we sought to establish the use of a novel protein biomarker, PrPC, within the blood plasma of rats exposed to simulated primary blast. This is the shockwave component of a blast, and is distinct from the other blast components that may cause injury, such as penetrating fragments (secondary), blast wind effects (tertiary), and noxious gases, heat, or dust (quaternary). Because of technical dif culties, the

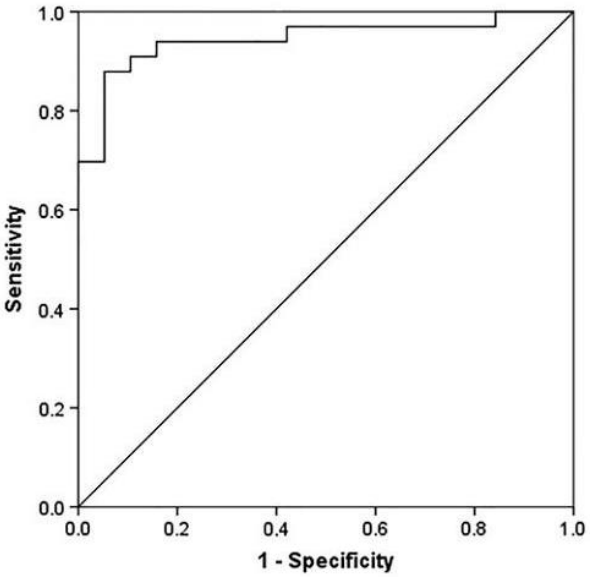

FIG. 5. Receiver operating characteristic (ROC) analysis of plasma soluble cellular prion protein (PrPC) for test of blast exposure. Control ($n = 19$) versus blast ($n = 33$) area under the curve (AUC) is 0.944 – 0.032 S.E. (95% CI, 0.881 1.000, $p < 0.0001$).

experimental replication of clean primary blast conditions has traditionally been problematic. However, recent developments in these laboratories have enabled the consistent replication of single pulse shockwaves with minimal concussive and whiplash forces (manuscript in preparation by Sawyer et al.), which is highly reminiscent of a free eld blast.[45]

We subjected adult male S-D rats to single pulse shockwaves of varying intensities, localized only to the head, in order to determine whether there was an appreciable rise in plasma PrPC concentration, which we quanti ed using a modi ed commercial ELISA kit. We hypothesized that the blast-induced shearing forces as described by Schardin may cause the predominantly extracellular, GPI-anchored PrPC to be dislodged from its neuronal lipid raft location.[55] Previous reports have shown increased plasma PrPC concentration following stroke, and in patients with various neurodegenerative diseases.[56,57] Additionally, there is a growing body of evidence reporting neurodegenerative changes post-TBI, which may possibly allude to an association with elevated plasma PrPC levels.[58 63]

In our current study, we have identi ed the rise in plasma levels of PrPC as a novel biomarker for detection of primary bTBI; and based on current literature search, this is the rst report of such an association. Statistical analysis determined that mean PrPC concentration in simulated primary blast exposed rats was signi cantly greater than in controls. Moreover, we also determined a mild positive correlation between plasma PrPC levels and increasing blast intensity (psi). Results showed dramatic increase of plasma PrPC in the 15 and 20 psi blast group, with levels plateauing at higher intensities. The reason or mechanism for this effect is not yet known, and will be the subject of further investigation, speci cally at blast exposure of lower intensity (< 15 psi), to better discern any functional relationship. In this regard, this initial nding suggests that subjects exposed to lower blast intensity elicit a similar plasma PrPC pro le to those at higher magnitudes. These ndings are in agreement with our recent immunohistochemical staining for neuro lament phosphorylation (unpublished data).

The translation of this nding to humans may mean that military service members exposed to primary blast waves only, including those at lower intensities, experience a similar effect to those exposed to waves at higher intensity, but may not receive medical attention because of lack of apparent injuries. Immunoblotting additionally con rmed, albeit semiquantitatively, that there is an apparent increase in plasma PrPC content after blast exposure compared with controls, which is consistent with quantitative ELISA results obtained. We determined the PrPC concentration cutoff value for blast exposure conservatively at 2.78 ng/mL (79.1% sensitivity and speci city, 81.6% PPV, and 85.7% NPV). It is noteworthy that there is currently no known standard reference database for normal rat plasma PrPC concentrations; therefore, the cutoff value determined is based on the normal concentration values that we have established.

In summary, our ndings support our working hypothesis that a primary blast force of suf cient intensity passing through brain tissue may dislodge the loosely attached PrPC from its extracellular domain, which subsequently accumulates within the systemic circulation.

The neuropathology of bTBI is not entirely clear, but reports have noted, among other symptoms, brain edema, cerebral pseudoaneurysms, intracerebral hemorrhaging, microlesions, cell death, and axonal injury as a result of blast exposure.[5,64 66] Such evidence establishes the basis that blast exposure can cause damage to brain tissue and vasculature. Furthermore, recent studies have shown that patients with cerebrovascular disease or vascular endothelial damage had higher levels of plasma PrPC than control values.[64 66] We cannot at this time discern whether our observation of increased plasma PrPC following primary blast exposure is exclusively of neural origin or if it also arises from the surrounding cerebrovasculature, which may also be subjected to primary blast-induced damage. Furthermore, PrPC has been reported to be upregulated following focal cerebral ischemia; therefore, it is possible that the rise in plasma PrPC content may be partially attributed to by damaged ischemic regions in the brain as a result of blast exposure.[67] Because the extent of blast-induced damage in the brain is unclear, it is possible that PrPC mRNA and protein expression may also be affected.

It is certain, however, that the rise in PrPC concentration is yet another part of the unique pathology complex associated with primary bTBI. In relation to primary bTBI, the neuroprotective function of PrPC may be of interest, as studies have noted its involvement in the context of hypoxia, epilepsy, oxidative stress, neurotoxicity, ischemic injury, and even in limiting brain damage in an animal model of TBI.[67 76] Further studies are needed to test our ndings in the acute period following primary bTBI to de nitively acknowledge it as a reliable biomarker, as well as to investigate the potential contributions of the other, non-primary aspects of bTBI (i.e., penetrating bodies, blunt trauma) to PrPC release, and its subsequent diagnostic utility.

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Author Disclosure Statement

No competing nancial interests exist.

References

1. Rao, V., and Lyketsos, C. (2000). Neuropsychiatric sequelae of traumatic brain injury. Psychosomatics 41, 95 103.
2. Cassidy, J.D., L.J., Carrol, L.J., Peloso, P.M., Borg, J., von Holst, H., Holm, L., Kraus, J., and Coronado, V.G. (2004). Incidence, risk factors and prevention of mild traumatic brain injury: results of the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. J. Rehabil. Med. S43, 28 60.
3. Rosenfeld, J.V., McFarlane, A.C., Bragge, P., Armonda, R.A., Grimes, J.B., and Ling, G.S. (2013). Blast-related traumatic brain injury. Lancet Neurol. 12, 882 893.
4. Menon, D.K., Schwab, K., Wright, D.W., and Mass, A.I. (2010). Position statement: de nition of traumatic brain injury. Arch. Phys. Med. Rehabil. 91, 1637 1640.
5. Ling, G., Bandak, F., Armonda, R., Grant, G., and Ecklund, J. (2009). Explosive blast neurotrauma. J. Neurotrauma 26, 815 825.
6. Hoge, C.W., McGurk, D., Thomas, J.L., Cox, A.L., Engel, C.C., and Castro, C.A. (2008). Mild traumatic brain injury in U.S. soldiers returning from Iraq. N. Engl. J. Med. 358, 453 456.
7. Defense and Veterans Brain Injury Center (2014). DoD Worldwide Numbers for TBI. Available at: http://www.dvbic.org/dod-worldwide-numbers-tbi Accessed May 1, 2013.
8. Hall, R.C., and Chapman, M.J. (2005). De nition, diagnosis, and forensic implications of postconcussive syndrome. Psychosomatics 46, 195 202.
9. Tanielian, T., and Jaycox, L.H. (2008). *Invisible Wounds of War: Psychological and Cognitive Injuries, Their Consequences and Services to Assist Recovery*. Rand Corp, MG 720-CCF: Santa Monica, CA.
10. Wolf, S.J., Bebarta, V.S., Bonnett, C.J., Pons, P.T., and Cantrill, S.V. (2009). Blast injuries. Lancet 374, 405 415.
11. Goldstein, L.E., Fisher, A.M., Tagge, C.A., Zhang, X., et al. (2012) Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model. Sci. Transl. Med. 4, 1 16.
12. Petrie, E.C., Cross, D.J., Yarnykh, V.L., Richards, T., Martin, N.M., Pagulayan, K., Hoff, D., Hart, k., Mayer, C., Tarabochia, M., Raskind, M.A., Minoshima, S., and Peskind, E.R. (2014). Neuroimaging, behavioral, and psychological sequelae of repetitive combined blast/impact mild traumatic brain injury in Iraq and Afghanistan war veterans. J. Neurotrauma 31, 425 436.
13. Elder, G.A., Mitsis, E.M., Ahlers, S.T., and Cristian, A. (2010). Blast-induced mild traumatic brain injury. Psychiatr. Clin. N. Am. 33, 757 781.
14. Wojcik, B.E., Stein, C.R., Bagg, K., Humphrey, R.J., and Orosco, J. (2010) Traumatic Brain Injury Hospitalization of U.S. Army Soldiers Deployed to Afghanistan and Iraq. Am. J. Prev. Med. 38, S108 S116.
15. Ramasamy, A., Hill, A.M., Hepper, A.E., Bull A.M., and Clasper, J.C. (2009). Blast mines: physics, injury mechanisms and vehicle protection. J. R. Army Med. Corps 155, 258 264
16. Warden, D. (2006). Military TBI during the Iraq and Afghanistan wars. J. Head Trauma Rehabil. 21, 398 402.
17. Okie, S. (2005) Traumatic brain injury in the war zone. N. Engl. J. Med. 352, 2043 2047.
18. Graner, J., Oakes, T.R., French, L.M., and Riedy, G. (2013). Functional MRI in the investigation of blast-related traumatic brain injury. Front. Neurol. 4, 1 18.
19. Champion, H.R., Holcomb, J.B., and Young, L.A. (2009). Injuries from explosions: physics, biophysics, pathology, and required research focus. J. Trauma 66, 1468 1477.
20. Hicks, R.R., Fertig, S.J., Desrocher, R.E., Koroshetz, W.J., and Pancrazio, J.J. (2010). Neurological effects of blast injury. J. Trauma 68, 1257 1263.
21. Nakagawa, A., Manley, G.T., Gean, A.D., Ohtani, K., Armonda, R., Tsukamoto, A., Yamamoto, H., Takayama, K., and Tominaga, T. (2011). Mechanisms of primary blast-induced traumatic brain injury: insights from shock-wave research. J. Neurotrauma 28, 1101 1119.
22. MacDonald, C.L., Johnson, A.M., Cooper, D., Nelson, E.C., and Brody, D.L. (2011). Detection of blast-related traumatic brain injury in U.S. military personnel. N. Engl. J. Med. 364, 2091 2100.
23. Agoston, D.V., Gyorgy, A., Eidelman, O., and Pollard, H.B. (2009). Proteomic biomarkers for blast neurotrauma: targeting cerebral edema, in ammation, and neuronal death cascades. J. Neurotrauma 26, 901 911.
24. Agoston, D.V., and Elsayed, M. (2012). Serum-based protein biomarkers in blast-induced traumatic brain injury spectrum disorder. Front. Neurol. 3, 1 10.
25. Kobeissy, F., Mondello, S., Tumer, N., Toklu, H.Z., Whidden, M.A., Kirichenko, N., Zhang, Z., Prima, V., Yassin, W., Anagli, J., Chandra, N., Svetlov, S., and Wang, K.K.W. (2013). Assessing neuro-systemic and behavioral components in the pathophysiology of blast-related brain injury. Front. Neurol. 4, 1 19.
26. Kochanek, P.M., Dixon, E.D., Shellington, D.K., Shin, S.S., and Jenkins, L.W. (2013). Screening of biochemical and molecular mechanisms of secondary injury and repair in the brain after experimental blast-induced traumatic brain injury in rats. J. Neurotrauma 30, 920 937.
27. Arun, P., Abu-Taleb, R., Oguntayo, S., Tanaka, M., Wang, Y., Valiyaveettil, M., Long, J.B., Zhang, Y., and Nambiar, M.P. (2013). Distinct patterns of expression of traumatic brain injury biomarkers after blast exposure: role of compromised cell membrane integrity. Neurosci. Lett. 552, 87 91.
28. Bendheim, P.E., Brown, H.R., Rudelli, R.D., et al. (1992). Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. Neurology 42, 149 156.
29. Moser, M., Colello, R.J., Pott, U., and Oesch, B. (1995). Developmental expression of the prion protein gene in glial cells. Neuron 14, 509 517.
30. Aguzzi, A., and Polymenidou, M. (2004) Mammalian prion biology: one century of evolving concepts. Cell 116, 313 327.
31. Sales, N., Rodolfo, K., Hassig, R., et al. (1998). Cellular prion protein localization in rodent and primate brain. Eur. J. Neurosci. 10, 2464 2471.
32. Martins, V.R., and Brentani, R.R. (2002). The biology of the cellular prion protein. Neurochem. Int. 41, 353 355.
33. Collinge, J., Whittington, M.A., Sidle, K.C.L., et al. (1994). Prion protein is necessary for normal synaptic function. Nature 370, 295 297.
34. Didonna, A. (2012). Prion protein and its role in signal transduction. Cell. Mol. Biol. Lett. 18, 209 230.
35. Brown, D.R., Qin, K., Herms, J.W., Madlung, A., Manson, J., Strome, R., Fraser, P.E., Kruck, T., von Bohlen, A., Schulz Schaeffer, W., Giese, A., Westaway, D., and Kretzschmar, H. (1997). The cellular prion protein binds copper in vivo. Nature 390, 684 687.
36. Watt, N.T., Taylor, D.R., Kerrigan, T.L., et al. (2012). Prion protein facilitates uptake of zinc into neuronal cells. Nat. Commun. 3, 1 12.
37. Singh, A., Kong, Q., Luo, X., Petersen, R.B., Meyerson, H., and Singh, N. (2009) Prion protein (PrP) knock-out mice show altered iron metabolism: a functional role for PrP in iron uptake and transport. PLoS One 4, 1 14.
38. Ford, M.J., Burton, L.J., Li, H., Graham, C.H., Frobert, Y., Grassi, J., Hall, S.M., and Morris, R.J. (2002). A marked disparity between the expression of prion protein and its message by neurones of the CNS. Neuroscience 111, 533 551.
39. Biasini, E., Tumbaugh, J.A., Unterberger, U., and Harris, D.A. (2012). Prion protein at the crossroads of physiology and disease. Trends Neurosci. 35, 92 103.
40. Shmerling, D., Hegyi, I., Fischer, M., et al. (1998). Expression of amino-terminally truncated PrP in the mouse leading to ataxia and speci c cerebellar lesions. Cell 93, 203 214.
41. Kuwahara C, Takeuchi AM, Nishimura T, Haraguchi K, et al. (1999). Prions prevent neuronal cell-line death. Nature 400, 255 256.
42. Coulpier, M., Messiaen, S., Boucreaux, D., and Elliot, M. (2006). Axotomy-induced motoneuron death is delayed in mice overexpressing PrPc. Neuroscience 141, 1827 1834.
43. Mitteregger, G., Vosko, M., Krebs, B., et al. (2007). The role of the octarepeat region in neuroprotective function of the cellular prion protein. Brain Pathol. 17, 174 183.
44. Carnini, A., Casha, S., Yong, V.W., Hurlbert, R.J., and Braun, J.E.A. (2010). Reduction of PrPC in human cerebrospinal uid after spinal cord injury. Prion 4, 80 86.
45. Ritzel, D.V., Parks, S.A., Roseveare, J., Rude, G., and Sawyer T.W. (2011). *Experimental Blast Simulation for Injury Studies*. NATO HFM 207: Halifax.

46. Taghibiglou, C., Lu, J., Mackenzie, I.R., Wang, Y.T., and Cashman, N.R. (2011). Sterol regulatory element binding protein-1 (SREBP1) activation in motor neurons in excitotoxicity and amylotrophic lateral sclerosis (ALS): Indip, a potential therapeutic peptide. Biochem. Biophys. Res. Commun. 413, 159 163.
47. Greiner, M., Sohr, D., and Gobel, P. (1995) A modi ed ROC analysis for the selection of cut-off values and the de nition of intermediate results of serodiagnostic tests. J. Immunol. Methods 185, 123 132.
48. Barkhoudarian, G., Hovda, D.A., and Giza, C.C. (2011). The molecular pathophysiology of concussive brain injury. Clin. Sports Med. 30, 33 48.
49. DeKosky, S.T., Ikonomovic, M.D., and Gandy, S. (2010). Traumatic brain injury football, warfare, and long-term effects. N. Engl. J. Med. 14, 1293 1296.
50. Unden, J., Bellner, J., Alling, C., Ingebrigtsen, T., and Rommer, B. (2005). Raised serum S100B levels after acute bone fractures without cerebral injury. J. Trauma 58, 59 61.
51. Savola, O., Pyhtinen, J., Leino, T.K., Siitonen, S., Niemela, O., and Hillbom, M. (2004). Effects of head and extracranial injuries on serum protein S100B levels in trauma patients. J. Trauma 56, 1229 1234.
52. Anderson, R.E., Hansson, L.O., Nilsson, O., Dijlai Merzoug, R., and Settergren, G. (2001). High serum S100B levels for trauma patients without head injuries. Neurosurgery 48, 1255 1258.
53. Harpio, R., and Einarsson, R. (2004). S100 proteins as cancer biomarkers with focus on S100B in malignant melanoma. Clin. Biochem. 37, 512 528.
54. Metting, Z., Wilczak, N., Rodiger, L.A., Schaaf, J.M., and van der Naalt, J. (2012). GFAP and S100B in the acute phase of mild traumatic brain injury. Neurology 78, 1428 1433.
55. Schardin, H. (1950). *The Physical Principles of the Effects of a Detonation. German Aviation Medicine, World War II.* Department of the US Air Force, Of ce of the Surgeon General: Washington DC.
56. Mitsios, N., Saka, M., Krupinski, J., Pennucci, R., Sanfeliu, C., Miguel, T.M., Gaffney, J., Kumar, P., Kumar, S., Sullivan, M., and Slevin, M. (2007). Cellular prion protein is increased in the plasma and peri-infarcted brain tissue after acute stroke. J. Neurosci. Res. 85, 602 611.
57. Volkel, D., Zimmermann, K., Zerr, I., Bodemer, M., Linder, T., Turecek, P.L., Poser, S., and Schwarz, H.P. (2001). Immunochemical determination of cellular prion protein in plasma from healthy subjects and patients with sporadic CJD or other neurologic diseases. Transfusion 41, 441 448.
58. Smith, D.H., Uryu, K., Saatman, K.E., Trojanowski, J.Q., and McIntosh, T.K. (2003). Protein accumulation in traumatic brain injury. Neuromolecular Med. 4, 59 72.
59. Uryu, K., Giasson, B.I., Longhi, L., Martinez, D., Murray, I., Conte, V., Nakamura, M., Saatman, K., Talbot, K., Horiguchi, T., McIntosh, T., Lee, V.M.Y., and Trojanowski, J.Q. (2003). Age-dependent synuclein pathology following traumatic brain injury in mice. Exp. Neurol. 184, 214 224.
60. Sidaros, A., Skimminge, A., Liptrot, M.G., Sidaros, K., Engberg, A.W. Herning, M., Paulson, O.B., Jemigan, T.L., and Rostrup, E. (2009). Long-term global and regional brain volume changes following severe traumatic brain injury: A longitudinal study with clinical correlates. NeuroImage 44, 1 8.
61. Stern, R.A., Riley, D.O., Daneshvar, D.H., Nowinski, C.J., Cantu, R.C., and McKee, A.C. (2011). Long-term consequences of repetitive brain trauma: chronic traumatic encephalopathy. Am. Acad of Phys. Med. Rehabil. 3, S460 S467.
62. Small, G.W., Kepe, V., Siddarth, P., Ercoli, L.M., Merrill, D.A., Donoghue, N., Bookheimer, S.Y., Martinez, J., Omalu, B., Bailes, J., and Barrio, J.R. (2013). PET scanning of brain TAU in retired national football league players: preliminary ndings. Am. J. Geriatr. Psychol. 2, 138 144.
63. Johnson, V.E., Stewart, J.E., Begbie, F.D., Trojanowski, J.Q., Smith, D.H., and Stewart, W. (2013). In ammation and white matter degeneration persist for years after a single traumatic brain injury. Brain 136, 28 42.
64. Krupinski, J., Turu, M.M., Lugue, A., Badimon, L., and Slevin, M. (2008). Increased PrPc expression correlates with endoglin (CD105) positive microvessels in advanced carotid lesions. Acta. Neuropathol. 116, 537 545.
65. Starke, R., Drummond, O., MacGregor, I., Biggerstaff, J., Gale, R., Camilleri, R., Mackie, I., Machin, S., and Harrison, P. (2002). The expression of prion protein by endothelial cells: a source of the plasma form of prion protein? Br. J. Haematol. 119, 863 873.
66. Simak, J., Holada, K., D Agnillo, F., Janota, J., and Vostal, J.G. (2002). Cellular prion protein is expressed on endothelial cells and is released during apoptosis on membrane microparticles found in human plasma. Transfusion 42, 334 342.
67. Weise, J., Crome, O., Sandau, R., Schulz Schaeffer, W., Bahr, M., and Zerr, I. (2004). Upregulation of cellular prion protein (PrPC) after focal cerebral ischaemia and in uence of lesion severity. Neurosci. Lett. 372, 146 150.
68. McLennan, N.F., Brennan, P.M., McNeil, A., Davies, I., et al. (2004). Prion protein accumulation and neuroprotection in hypoxic brain damage. Am. J. Pathol. 165, 227 235.
69. Walz, R., Amaral, O.B., Rockenbach, I.C., et al. (1999). Increased sensitivity to seizures in mice lacking cellular prion protein. Epilepsia 40, 1679 1682.
70. Milhavet, O., McMahon, H.E.M., Rachidi, W., Nishida, N., Katamine, S., Mange, A., Arlotto, M., Casanova, D., Riondel, J., Favier, A., and Lehmann, S. (2000) Prion infection impairs the cellular response to oxidative stress. Proc. Natl. Acad. Sci. U.S.A. 97, 13,937 13,942.
71. Rangel, A., Burgaya, F., Gavin, R., Soriano, E., Aguzzi, A., and del Rio, J.A. (2007). Enhanced susceptibility of Prnp-de cient mice to kainite-induced seizures, neuronal apoptosis, and death: Role of AMPA/kainite receptors. J. Neurosci. Res. 85, 2741 2755.
72. You, H., Tsutsui, S., Hameed, S., et al. (2012). Aβ neurotoxicity depends on interactions between copper ions, prion protein, and N-methyl-D-aspartate receptors. Proc. Natl. Acad. Sci. U.S.A. 109, 1737 1742.
73. Weise, J., Sandau, R., Schwarting, S., et al. (2006). Deletion of cellular prion protein results in reduced Akt activation, enhanced post-ischemic caspase-3 activation, and exacerbation of ischemic brain injury. Stroke 37, 1296 1300.
74. Shyu, W.C., Lin, S.Z., Chiang, M.F., Ding, D.C., Li, K.W., Chen, S.F., Yang, H.I., and Li, H. (2005). Overexpression of PrPC by adenovirus-mediated gene targeting reduces ischemic injury in a stroke rat model. J. Neurosci. 25, 8967 8977.
75. Spudich, A., Frigg, R., Kilic, E., et al. (2005) Aggravation of ischemic brain injury by prion protein de ciency: role of ERK-1/-2 and STAT-1. Neurobiol. Dis. 20, 442 449.
76. Hoshino, S., Inoue, K., Yokoyama, T., Kobayashi, S., Asakura, T., Teramoto, A., and Itohara, S. (2003). Prions prevent brain damage after experimental brain injury: a preliminary report. Acta Neurochir. S86, 297 299.

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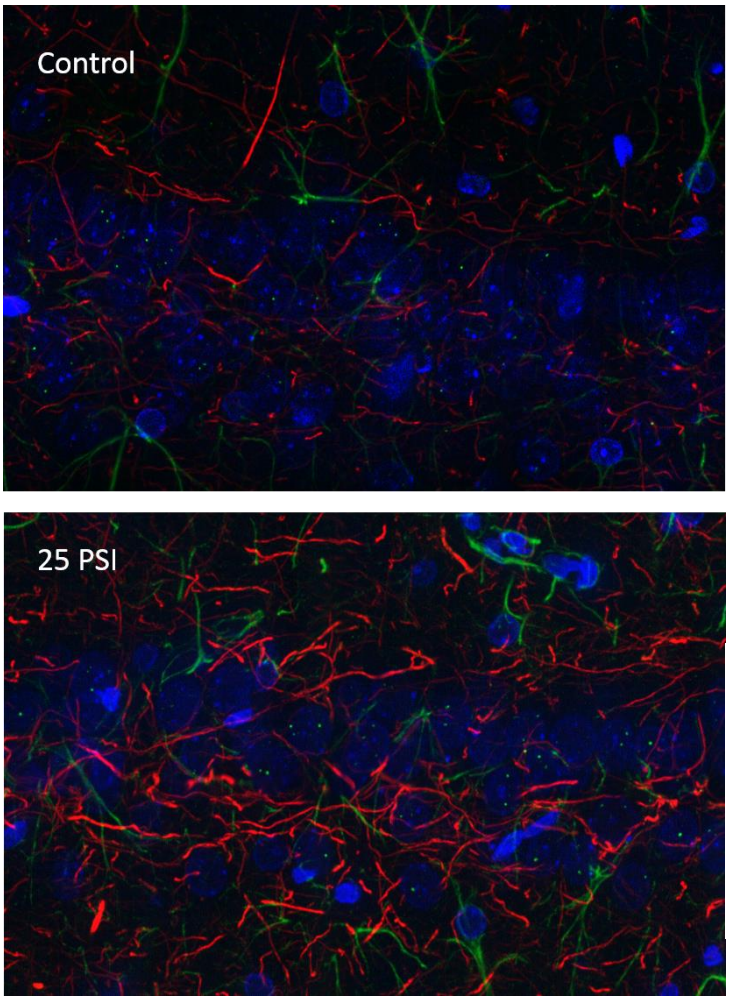
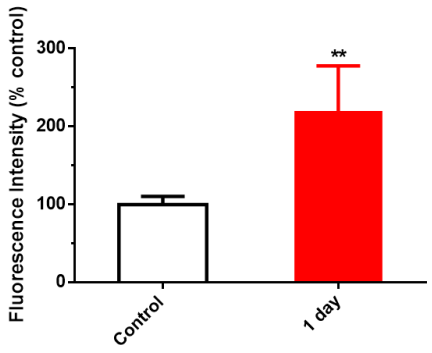
5.2. Neuronal Dysregulation following Blast

To show the presence of neuronal damage, immunohistochemical fluorescence staining was performed by Dr. Yushan Wang (DRDC) on neurofilament heavy chain (NFH) and GFAP. Neurofilaments are CNS-specific structural components of neurons that determine size and shape, and are also very abundant in axons as they are involved in maintaining axonal size, caliber, and nerve impulse conduction (Yuan, et al. 2012). In various disease or neurological states involving neuronal and axonal degeneration or injury, including TBI, there is hyperphosphorylation of NFH (pNFH) (Shaw, et al. 2005). Similarly, GFAP is also upregulated within astrocytes following neuronal injury and axonal denervation due to TBI (Burbach, et al. 2004). To show that blast exposure from 15-30 PSI is sufficient to cause neuronal injury, coronal brain sections containing the hippocampus, ipsilateral to the side of blast exposure, were prepared and stained for NFH (both normal and phosphorylated forms) and GFAP using fluorescent immunohistochemical techniques. pNFH was indeed increased in blast exposed sections just over two-fold in the CA1 and dentate gyrus section of the hippocampus compared to sham controls (**see figures 5.1 and 5.2**). Conversely, immunoreactivity to non-phosphorylated NFH within these regions was not significantly different (**see figure 5.3 and 5.4**). Furthermore, GFAP staining also showed no upregulation in either hippocampal region (**see figure 5.3 and 5.4**). This remains in line with previous findings showing that GFAP may not be robustly changed following mTBI in sports, which is very similar to bTBI (Neselius, et al. 2012; Zetterberg, et al. 2006). It is also possible that GFAP upregulation may not have occurred at 24 hours following injury; further testing

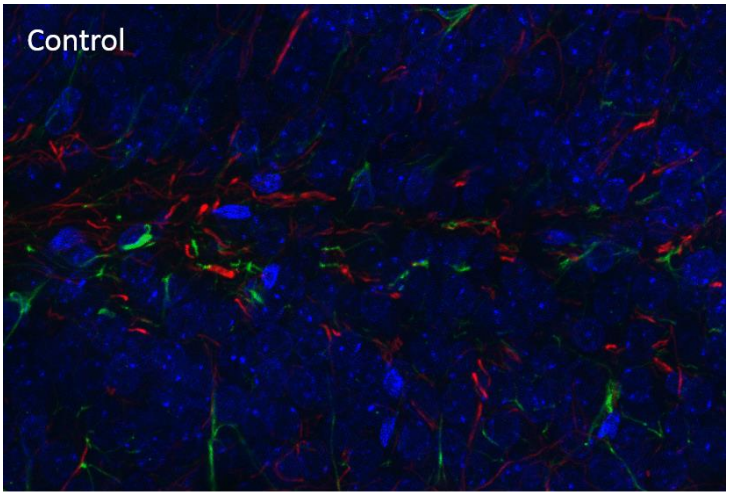
Figure 5.1. Immunofluorescence of pNFH-GFAP-DAPI in CA1. One day following blast exposure (25 PSI; n=4) there is increased pNFH (red) in the CA1 region of the ipsilateral hippocampus by 2.18 fold compared to sham controls (0 PSI; n=4). T-test shows that relative pNFH immunofluorescence intensity is significantly higher in blast (218.0 ± 57.0%) compared with normalized controls (100.0 ± 9.3%) (p<0.001).

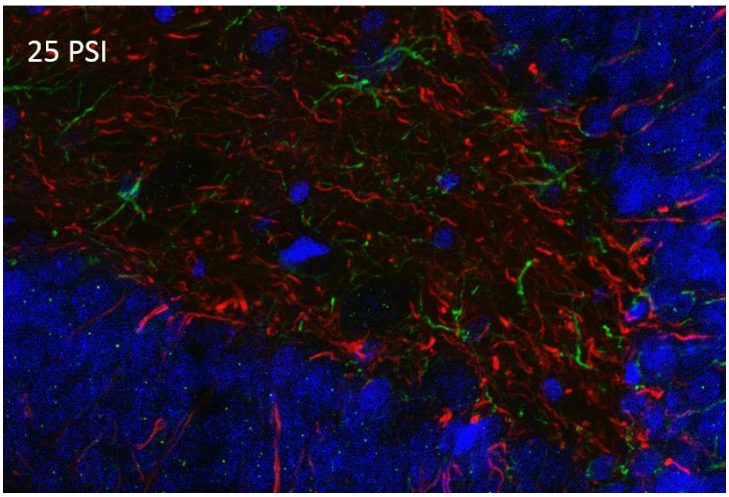


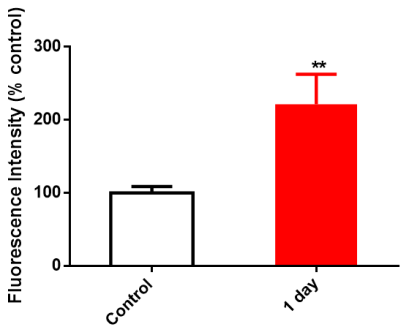


Relative fluorescence intensity of p-NFH in the CA1 region of ipsilateral hippocampus in rats exposed to 25 PSI blast

Figure 5.2. Immunofluorescence of pNFH-GFAP-DAPI in DG. One day following blast exposure (25 PSI; n=4) there is increased pNFH (red) in the dentate gyrus area of the ipsilateral hippocampus by 2.2 fold compared to sham controls (0 PSI; n=4). T-test shows that relative pNFH immunofluorescence intensity is significantly higher in blast (220.0 ± 40.5%) compared with normalized control (100 ± 9.5%) (p<0.001).

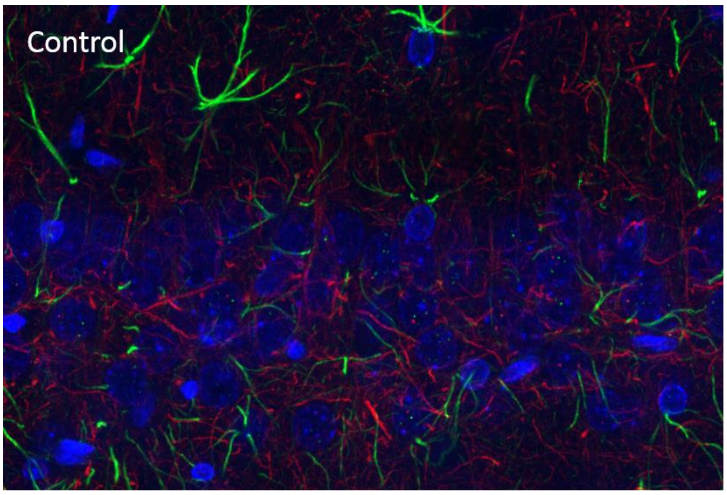


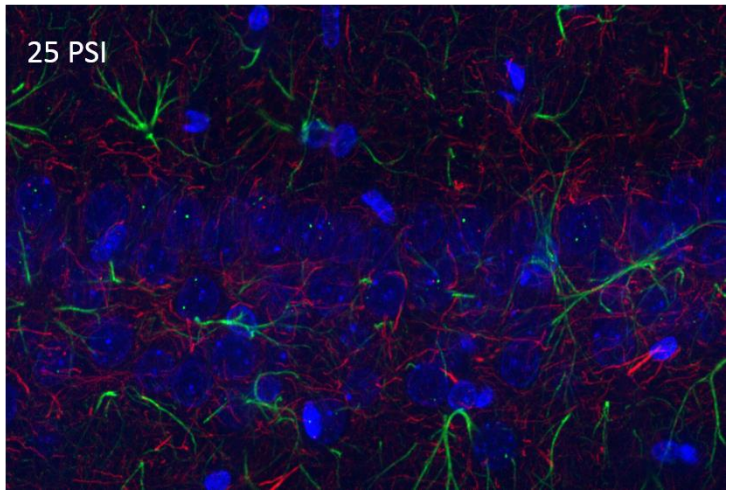


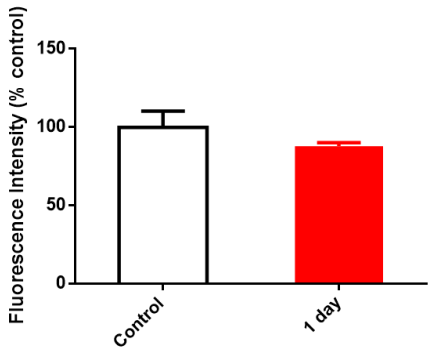


Relative fluorescence intensity of p-NFH in the Dentate Gyrus area of ipsilateral hippocampus in rats exposed to 25 PSI blast

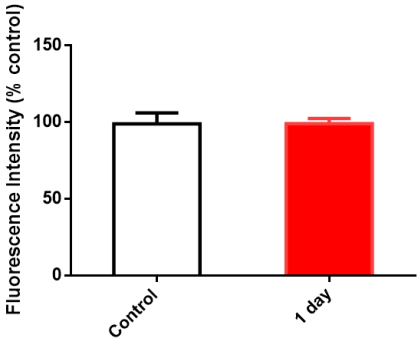
Figure 5.3. Immunofluorescence of NFH-GFAP-DAPI in CA1. One day following blast exposure (25 PSI; n=4) there is no change in NFH (red) and GFAP (green) in the CA1 region of the ipsilateral hippocampus compared to sham controls (0 PSI; n=4). T-test shows that relative NFH immunofluorescence is not significantly different in blast (87.0 ± 3.8%) compared with normalized control (100.0 ± 10.8%) (p>0.05). Similarly, relative GFAP immunofluorescence is not significantly different in blast (99.0 ± 3.3%) compared with normalized control (100.0 ± 7.8%) (p>0.05).





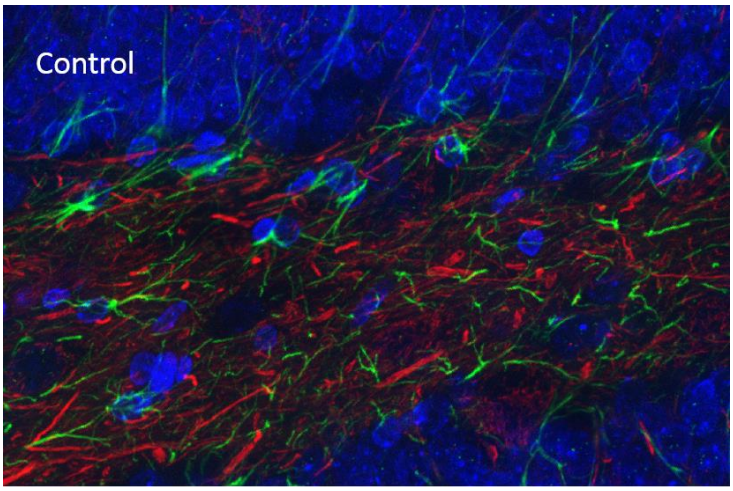


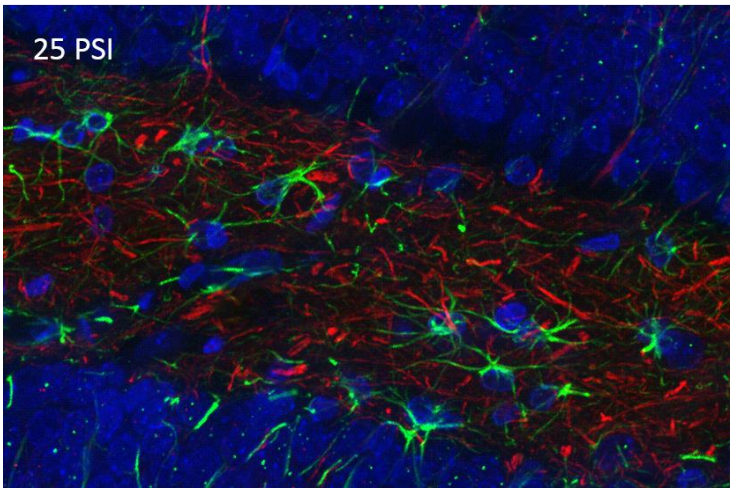
Relative fluorescence intensity of NFH in the CA1 region of ipsilateral hippocampus in rats exposed to 25 PSI blast

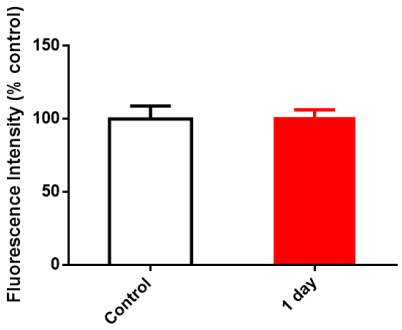


Relative fluorescence intensity of GFAP in the CA1 region of ipsilateral hippocampus in rats exposed to 25 PSI blast

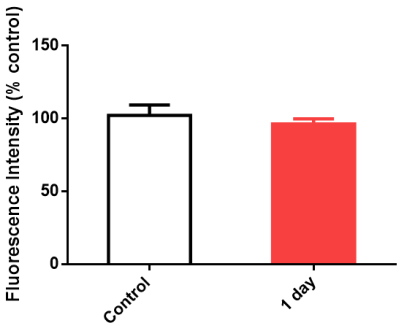
Figure 5.4. Immunofluorescence of NFH-GFAP-DAPI in DG. One day following blast exposure (25 PSI; n=4) there is no change in NFH (red) and GFAP (green) in the dentate gyrus area of the ipsilateral hippocampus compared to sham controls (0 PSI; n=4). T-test shows relative NFH immunofluorescence intensity is not significantly different in blast (100.5 ± 5.5%) compared with normalized control (100.0 ± 8.0%) (p>0.05). Similarly, relative GFAP immunofluorescence is not significantly different in blast (96.5 ± 1.3%) compared with normalized control (100.0 ± 7.7%) (p>0.05).







Relative fluorescence intensity of NFH in the Dentate Gyrus area of ipsilateral hippocampus in rats exposed to 25 PSI blast



Relative fluorescence intensity of GFAP in the Dentate Gyrus area of ipsilateral hippocampus in rats exposed to 25 PSI blast

5.3. Plasma PrP^C Results.

5.3.1. Multiple Conditions Comparison

As shown in published work above, (**see Section 5.1**) pooled blast group data did not show normal distribution and non-parametric methods were used. One-way ANOVA showed that different blast intensity exposure data sets differed significantly in distribution from restraint and whiplash, but not in net (**see Figure 5.5**). Post-hoc Dunnett's multiple comparison test for restraint group results shows that 15, 20, and 25 PSI were indeed significantly different than controls, but not for 30 PSI. Post-hoc comparison of whiplash group results show that only 20 PSI was significantly different than control. And post-hoc comparison of net group results showed no significantly different pairing against control. For results summary of plasma PrP^C concentrations **see Table 5.1.**

5.3.2. Long-term plasma PrP^C time course

Comparison of plasma PrP^C up to four weeks following blast exposure was also performed to determine the long-term change in plasma protein concentration (**see Figure 5.5**). Plasma samples were collected from restraint group S-D rats exposed to 25 PSI at different time points following exposure between 1-28 days. Samples were not serially collected from the same animals over time, but instead were collected through decapitation for different sets of animals at each time point. Kruskal-Wallis ANOVA shows that plasma PrP^C is significantly elevated compared to sham controls 1 day after exposure. PrP^C remains slightly elevated up to one week, but returns to control levels by two weeks and beyond

5.3.3 Western Blotting of TBI biomarkers

Western blot analysis for animals in restraint showed a marked increase in immunoreactivity (IR) for protein bands of PrP^C, ADAM10, and ApoE at higher blast magnitudes at 25 and 30 PSI compared to sham controls (**see Figure 5.7**). PrP^C IR showed a blast intensity-dependent increase trend in all blast groups, but was only statistically significant to controls at higher intensities. Immunoblotting for whiplash group shows that PrP^C IR is similarly increased in plasma following blast exposure (**see Figure 5.8**). ADAM10 and ApoE were also increased in whiplash group with increasing intensity, but not to the same degree as in restraint group animals. Net group showed a slight upward trend in protein IR for all markers, but none were significantly elevated compared to controls (**see Figure 5.9**).

Table 5.1. PrP^C ELISA Results Summary

			Plasma PrP^C Concentration (ng/mL)		
Group	**PSI**	**n**	**Mean ± SD**	**Median**	**Range**
Sham Controls	0	19	2.46 ± 0.63	2.66	0.67—3.35
Restraint	15	7	3.74 ± 0.89	3.99	2.10—4.67
	20	7	4.27 ± 0.69	4.47	3.27—5.34
	25	12	4.18 ± 0.63	4.26	3.06—5.37
	30	7	3.54 ± 0.79	3.25	2.68—4.84
Whiplash	15	2	3.21 ± 0.51	3.32	2.96—3.70
	20	3	4.10 ± 0.33	4.09	3.78—4.10
	25	3	3.17 ± 0.09	3.17	3.09—3.26
	30	7	3.13 ± 0.73	2.92	2.53—3.94
Net	15	3	3.11 ± 0.20	3.17	2.89—3.28
	20	3	2.54 ± 0.18	2.58	2.35—2.71
	25	3	2.34 ± 0.30	2.17	2.16—2.69
	30	4	3.09 ± 0.54	3.29	2.30—3.48

Figure 5.5. Comparison of plasma PrP^C in blast groups. One-way ANOVA of plasma PrP^C from different blast intensity exposed groups showed results were not equally distributed for restraint (p<0.0001) and whiplash (p=0.0013) groups, but not significant in net (p=0.1374). Post-hoc Dunnett's multiple comparison test of blast groups vs control for **restraint** group is as follows: 15 PSI (p<0.001), 20 PSI (p<0.001), 25 PSI (p<0.001), and 30 PSI (p<0.01). Post-hoc Dunnett's multiple comparison test of blast groups vs control for **whiplash** group showed only 20 PSI groups results were significantly different from control (p<0.001). Post-hoc Dunnett's multiple comparison test of blast groups vs control for **net** group showed that none of the groups were significantly different compared to control (p>0.05). *Note: see Table 5.1 for concentration values.*

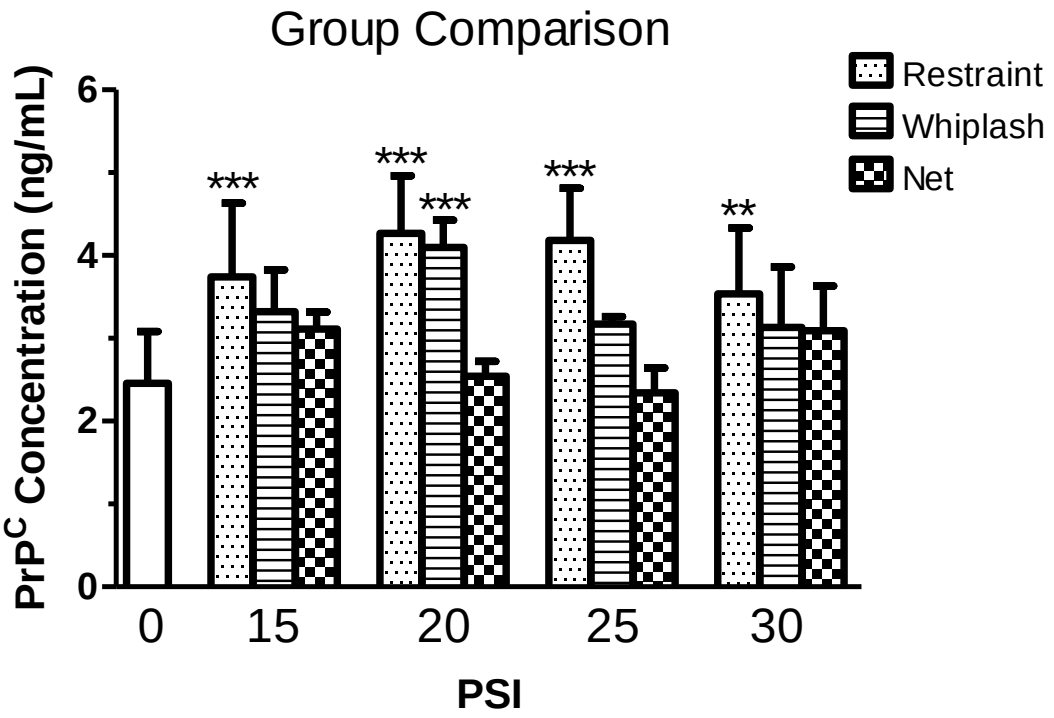


Figure 5.6. Long-term plasma PrP^C comparison. One-way ANOVA found that plasma PrP^C concentration in 25 PSI blast exposed animals at different time points was significantly different in distribution compared to non-blast exposed controls ($p<0.0001$). Post-hoc Dunnett's multiple comparison test of plasma PrP^C at different time points are as follows: 1 day (n=12, mean 4.18 ± 0.63 ng/mL; $p<0.001$), 4 days (n=3, mean 3.54 ± 0.38 ng/mL; $p<0.05$), 7 days (n=4, mean 2.97 ± 0.47 ng/mL; NS), 14 days (n=4, mean 2.56 ± 0.87 ng/mL; NS), 28 days (n=3, mean 2.39 ± 0.66Gr ng/mL; NS), and control (n=19, mean 2.46 ± 0.63 ng/mL).

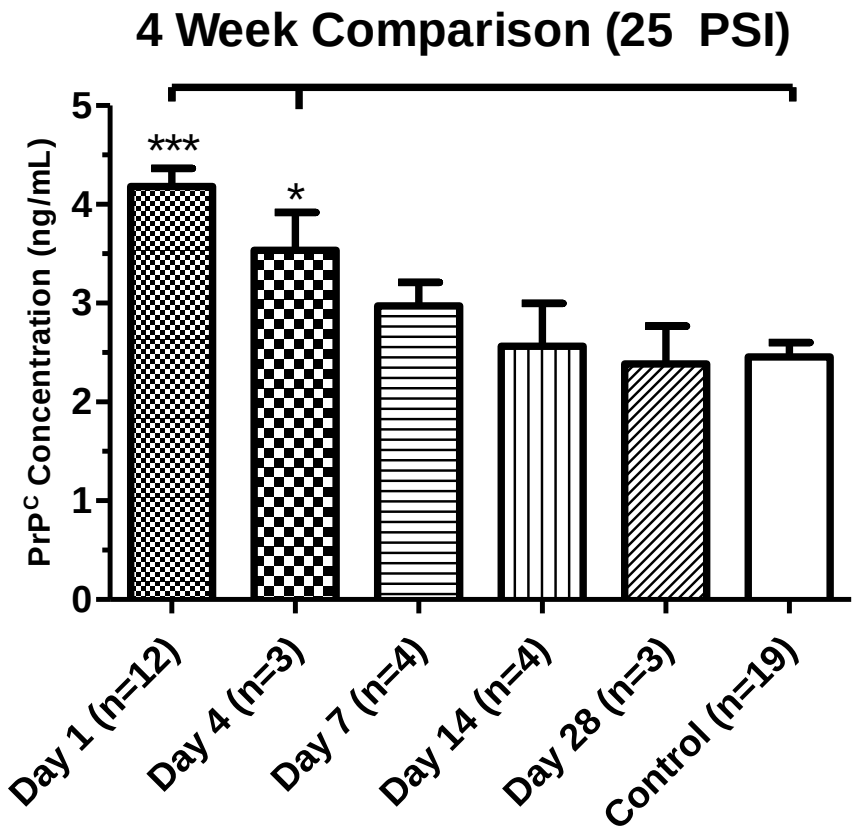


Figure 5.7. Restraint western blotting. One-way ANOVA found that mean IR of target proteins in blast exposed groups was significantly different relative to control for PrP^C ($p<0.05$), ADAM10 ($p<0.001$), and ApoE ($p<0.01$). Post-hoc Dunnett's multiple comparison of blast groups against control for PrP^C IR are as follows: 0 PSI (n=5, mean 1.00 ± 0.79 fold), 15 PSI (n=4, mean 1.78 ± 0.42 fold), 20 PSI (n=4, mean 1.52 ± 0.48 fold), 25 PSI (n=4, mean 2.25 ± 0.45 fold; $p<0.05$), and 30 PSI (n=4, mean 2.42 ± 1.78 fold; $p<0.05$). Post-hoc comparison of ADAM10 IR are as follows: 0 PSI (n=5, mean 1.00 ± 0.71 fold), 15 PSI (n=4, mean 1.32 ± 0.57 fold), 20 PSI (n=4, mean 1.59 ± 0.72 fold), 25 PSI (n=4, mean 2.79 ± 0.59 fold; $p<0.01$), and 30 PSI (n=4, mean 3.77 ± 0.85 fold; $p<0.001$). Post-hoc comparison of ApoE are as follows: 0 PSI (n=5, mean 1.00 ± 0.03 fold), 15 PSI (n=4, mean 1.88 ± 0.63 fold), 20 PSI (n=4, mean 1.39 ± 0.19 fold), 25 PSI (n=4, mean 3.03 ± 1.23 fold), and 30 PSI (n=4, mean 5.06 ± 1.42 fold; $p<0.01$). *Note: asterisks designate significance compared to corresponding control column.*

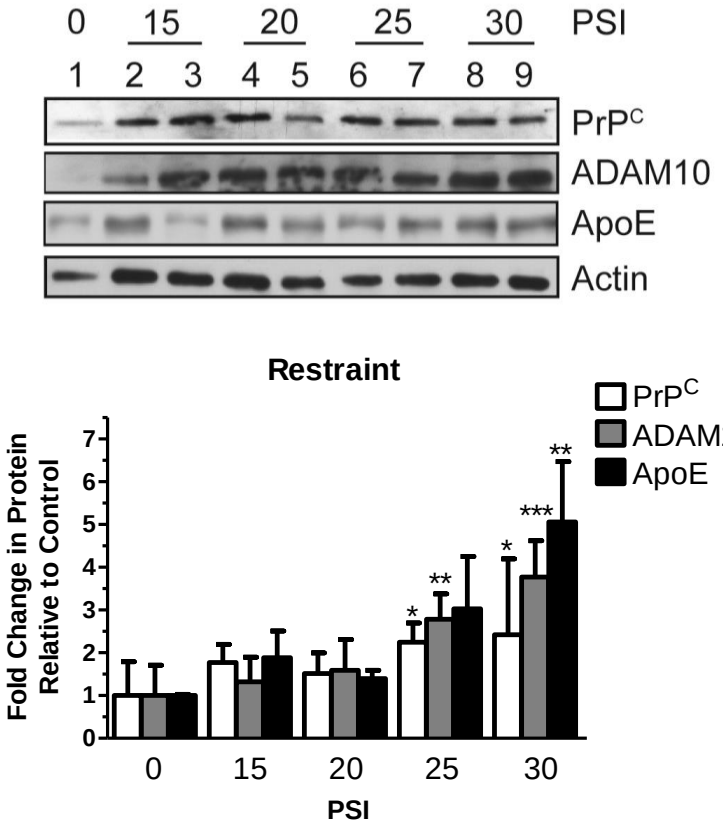


Figure 5.8. Whiplash western blotting. One-way ANOVA found that mean IR of PrP^C in blast exposed groups was significantly different relative to control (p<0.01), but was not significant for ADAM10 (p>0.05), and ApoE (p=0.0502). Post-hoc Dunnett's multiple comparison of blast groups against control for PrP^C IR are as follows: 0 PSI (n=5, mean 1.00 ± 0.79 fold), 15 PSI (n=2, mean 2.14 ± 1.32 fold), 20 PSI (n=3, mean 2.64 ± 1.17 fold), 25 PSI (n=3, mean 3.40 ± 0.76 fold; p<0.01), and 30 PSI (n=3, mean 3.14 ± 1.04 fold; p<0.01). Post-hoc comparison of ADAM10 IR are as follows: 0 PSI (n=5, mean 1.00 ± 0.71 fold), 15 PSI (n=2, mean 1.51 ± 0.43 fold), 20 PSI (n=3, mean 1.59 ± 0.32 fold), 25 PSI (n=3, mean 1.89 ± 0.04 fold), and 30 PSI (n=3, mean 2.25 ± 0.07 fold; p<0.05). Post-hoc comparison of ApoE are as follows: 0 PSI (n=3, mean 1.00 ± 0.81 fold), 15 PSI (n=2, mean 1.71 ± 0.28 fold), 20 PSI (n=3, mean 2.04 ± 0.60 fold), 25 PSI (n=3, mean 1.80 ± 0.20 fold), and 30 PSI (n=3, mean 2.62 ± 0.48 fold; p<0.05).
Note: asterisks designate significance compared to corresponding control column.

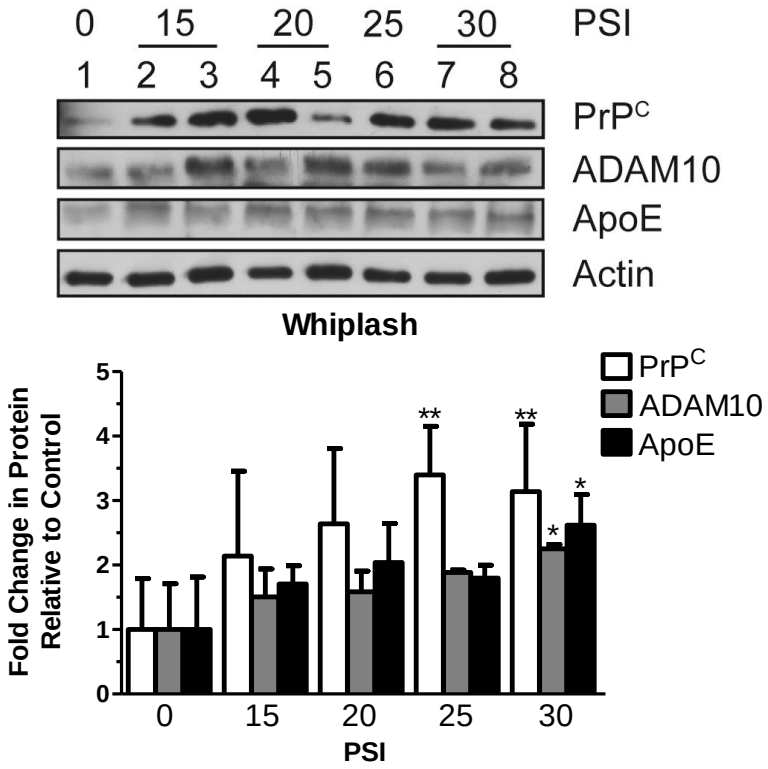
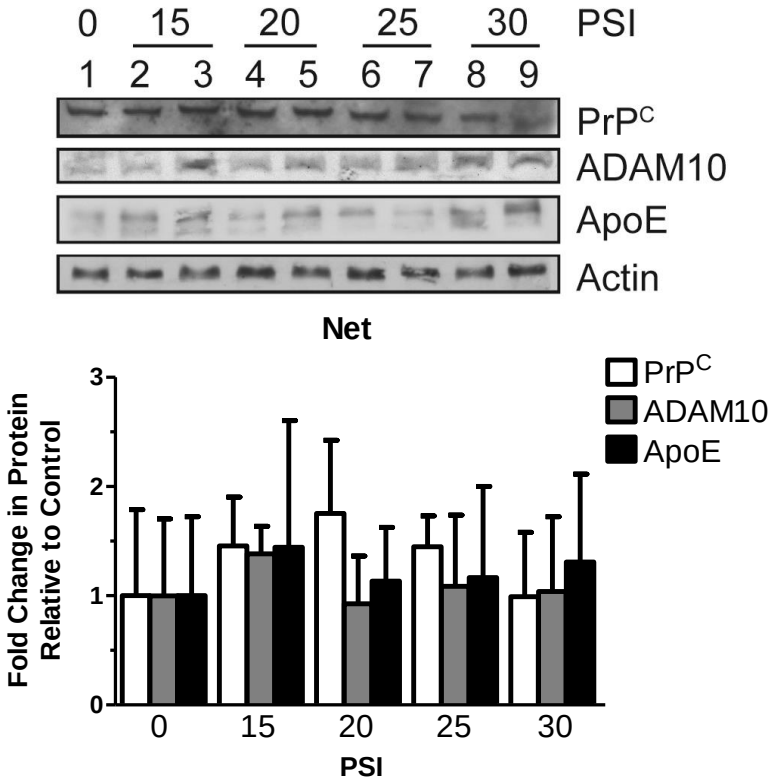


Figure 5.9. Net western blotting. One-way ANOVA found that mean IR of target proteins in blast exposed groups was not significantly different relative to control for PrP^C, Adam10, and ApoE (p>0.05). Post-hoc Dunnett's multiple comparison of blast groups against control for PrP^C IR are as follows: 0 PSI (n=5, mean 1.00 ± 0.79 fold), 15 PSI (n=3, mean 1.46 ± 0.45 fold), 20 PSI (n=3, mean 1.75 ± 0.67 fold), 25 PSI (n=3, mean 1.45 ± 0.28 fold), and 30 PSI (n=3, mean 0.99 ± 0.59 fold). Post-hoc comparison of ADAM10 IR are as follows: 0 PSI (n=5, mean 1.00 ± 0.71 fold), 15 PSI (n=3, mean 1.39 ± 0.25 fold), 20 PSI (n=3, mean 0.93 ± 0.44 fold), 25 PSI (n=3, mean 1.09 ± 0.65 fold), and 30 PSI (n=3, mean 1.04 ± 0.69 fold). Post-hoc comparison of ApoE are as follows: 0 PSI 0 PSI (n=3, mean 1.00 ± 0.73 fold), 15 PSI (n=3, mean 1.44 ± 1.16 fold), 20 PSI (n=3, mean 1.14 ± 0.49 fold), 25 PSI (n=3, mean 1.17 ± 0.84 fold), and 30 PSI (n=3, mean 1.31 ± 0.81 fold). *Note: asterisks designate significance compared to corresponding control column.*



Section 6. Human Study Results

6.1. Establishing normal plasma PrP^C concentration in Healthy Adults

In order to investigate the possibility that the plasma level of PrP^C rises following mTBI, normal soluble PrP^C levels were first measured in the general population aged 18 years and over (mostly U of S students) without significant confounds due to illness, health condition, or significant head injury within the past six months. T-test comparison between male vs. female results showed no significant difference in mean concentration of plasma PrP^C, although female concentration was slightly higher (**see Figure 6.1)**. Additionally, we found a slight significant difference in mean plasma PrP^C between off season athletes' baselines vs. normal non-athlete students (**see Figure 6.1**).

Furthermore, aggregate results were separated into five age groups to determine any significant difference in plasma PrP^C across different age brackets (**see Figure 6.2**). One-way ANOVA for determining variation between mean plasma PrP^C concentration across age groups showed no significant difference.

6.2. Plasma soluble PrP^C concentration increases in Concussed Athletes

During the 2013-2014 season, the Huskies Athletic teams had 4 female and 2 male participants within the study who had suffered injuries causing concussion while engaging in various sports including Canadian football, ice hockey, basketball and wrestling. Initial signs and symptoms following injury were evaluated for the six athletes along a scale using the SCAT3 criteria by teams' doctors and physical therapists (**see Table 6.1**). The SCAT3 criteria is a combination of the GCS, cognitive ability tests, and self-reporting of symptoms that help determine the degree of injury. Individuals who identify themselves as having a symptom such

as dizziness, confusion, or being nauseous score these symptoms from a scale of 1-6 depending on increasing severity. For almost all incidents, the injuries sustained by the athletes involved a significant blow to the head and/or included rapid whiplash acceleration of the head along the neck. Depending on access and appropriate convenience to the concussed athletes, their blood samples were collected within 24hrs to 7 days post-mTBI. T-test comparison of mean plasma PrP^C in post-concussion samples was found to be significantly higher than levels in baseline samples collected during the offseason and against combined baselines with the normal population (**see Figure 6.3.A**). Of the 76 baseline samples collected from athlete participants during the offseason, only three individuals sustained a concussion during the season to allow pre- and post-TBI comparison (**see Figure 6.3.B**). Paired t-test comparison shows there was no significant difference between the three sets of pre- and post-TBI PrP^C values, although our data showed an increasing trend. For participant description and plasma PrPC results summary, **see Table 6.2.**

6.2.1. Western blotting of human plasma TBI biomarkers

Comparative western blotting was performed on plasma samples from injured athletes and age- and gender- matched controls. T-test comparison of IR for target proteins PrP^C, ADAM10, and 14-3-3 were increased in post-concussion samples compared to combined baseline and normal population samples, but only PrP^C and 14-3-3 were considered significant. *Note: two of the post-TBI samples were excluded for Western blotting because of the prolonged period between injury and collection (seven days).*

Figure 6.1. Group comparison of plasma PrP^C. Two-tailed unpaired student's t-test shows no significant difference between male (n=54; 1.63 ng/mL ± 0.10 SEM) and female (n=49; 1.79 ng/mL ± 0.10 SEM) (*p>0.05*). *T* test of athletes (n=76, 1.59 ng/mL ± 0.07 SEM) vs. the normal non-athlete population (n=27; 2.02 ng/mL ± 0.15 SEM) shows significant difference between mean PrP^C concentrations (*p<0.01*).

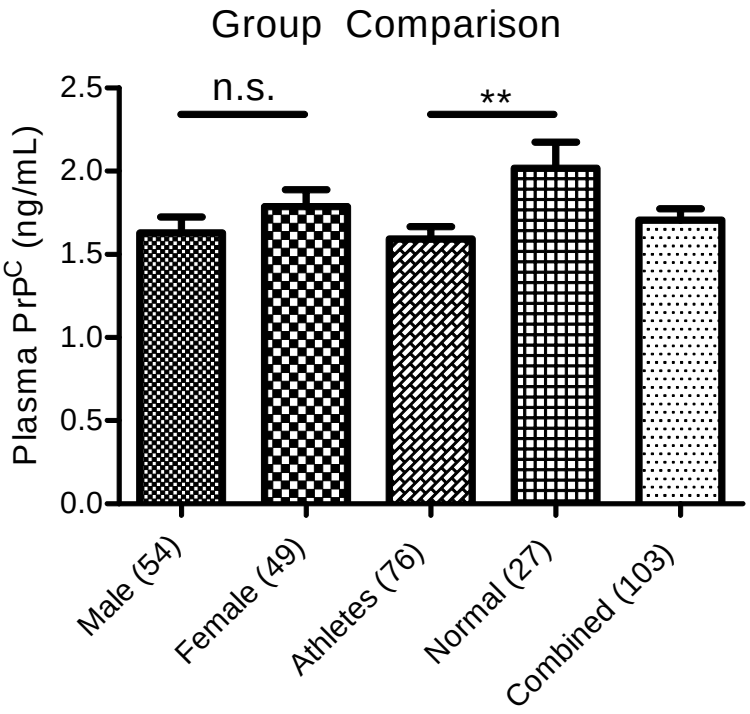


Figure 6.2. **Comparison of plasma PrP^C with age.** One-way ANOVA of PrP^C concentrations for different age groups shows there is no significant difference between mean concentrations for subjects between the ages of 18-20 (n=51; 1.66 ng/mL ± 0.68 SD), 21-33 (n=33; 1.61 ng/L ± 0.58 SD), 24-26 (n=14; 1.89 ng/mL ± 0.78 SD), 27-29 (n=4; 2.13 ng/mL ± 0.51 SD), and those 30 and over (n=9; 1.73 ng/mL ± 0.90 SD) (*p>0.05*).

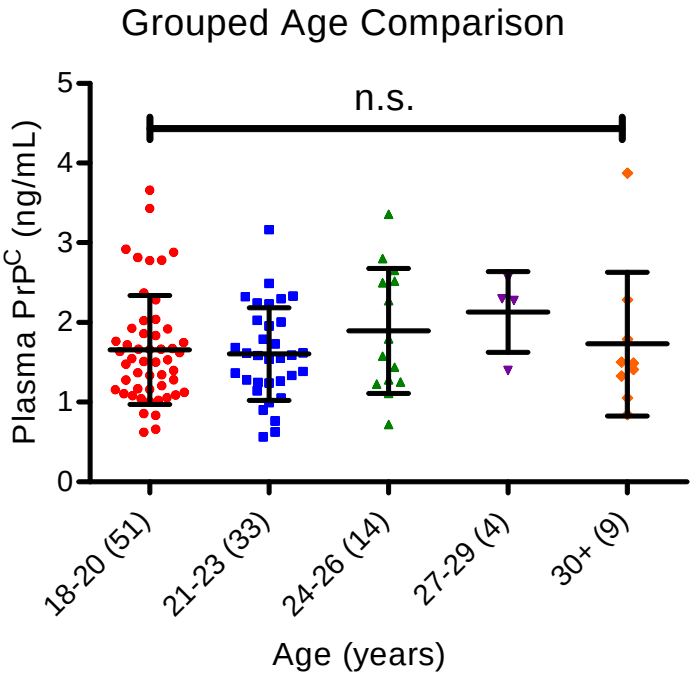


Table 6.1. Description of participants' head injury

#ID	Method of Injury	Initial Signs & Symptoms				
		LOC	Balance	Cognitive	Symptom Scale (#symptoms/total score)	Time from Injury to Collection
HMF27	Helmet to helmet contact	No	Yes	Yes	21/81	1 day
HMF28	Unknown	No	No	No	16/46	5 days
HFB15	Elbow to head	No	No	Yes	14/39	6 days
HFH13	Collision with player head on but no head contact, whiplash	No	Yes	No	6/13	2 days
HFW1	Punch to face	No	No	No	12/23	7 days
HFW2	Knee to temple, punch to head	No	Yes	No	19/71	7 days

LOC = Loss of Consciousness

Table 6.2. Plasma PrP^C in adult humans

Participant Summary

		Age (years)			PrP^C Concentration (ng/mL)		
	n	Mean ± SD	Median	Range	Mean ± SEM	Median	Range
Non-Athlete	**27**	**24.48 ± 2.99**	**24.00**	**18—30**	**2.02 ± 0.15**	**2.23**	**0.72—3.87**
Male	15	24.67 ± 1.76	24.00	22—29	2.12 ± 0.18	2.32	1.11—3.41
Female	12	24.25 ± 4.14	23.50	18—30	1.89 ± 0.27	2.27	0.72—3.87
Athlete	**76**	**20.04 ± 1.84**	**20.00**	**18—26**	**1.59 ± 0.64**	**1.51**	**0.56—3.66**
Male	39	20.41 ± 1.92	20.00	18—24	1.44 ± 0.10	1.34	0.56—3.17
Female	37	19.65 ± 1.70	19.00	18—26	1.75 ± 0.10	1.59	1.05—3.66
Combined	**103**	**21.20 ± 2.94**	**21.00**	**18—30**	**1.70 ± 0.07**	**1.55**	**0.56—3.87**
Male	54	21.59 ± 2.67	22.00	18—29	1.63 ± 0.10	1.40	0.56—3.41
Female	49	20.78 ± 3.18	20.00	18—30	1.79 ± 0.10	1.62	0.72—3.87

SD = Standard Deviation

SEM = Standard Error of the Mean

Figure 6.3. Comparison between normal and post-concussion. A) Two-tailed unpaired student's t-test shows post-TBI PrP^C concentrations (n=6; mean 2.96 ng/mL ± 0.37 SEM) are significantly elevated compared with either offseason athlete baseline concentration (n=76, mean 1.59 ng/mL ± 0.07 SEM)(p<0.0001), or both athletes and non-athletes combined (n=103; mean 1.70 ng/mL ± 0.07 SEM)(p<0.0001). **B)** Two-tailed paired t-test shows there was no significant difference between three sets of pre- and post-TBI PrP^C values (p>0.05)

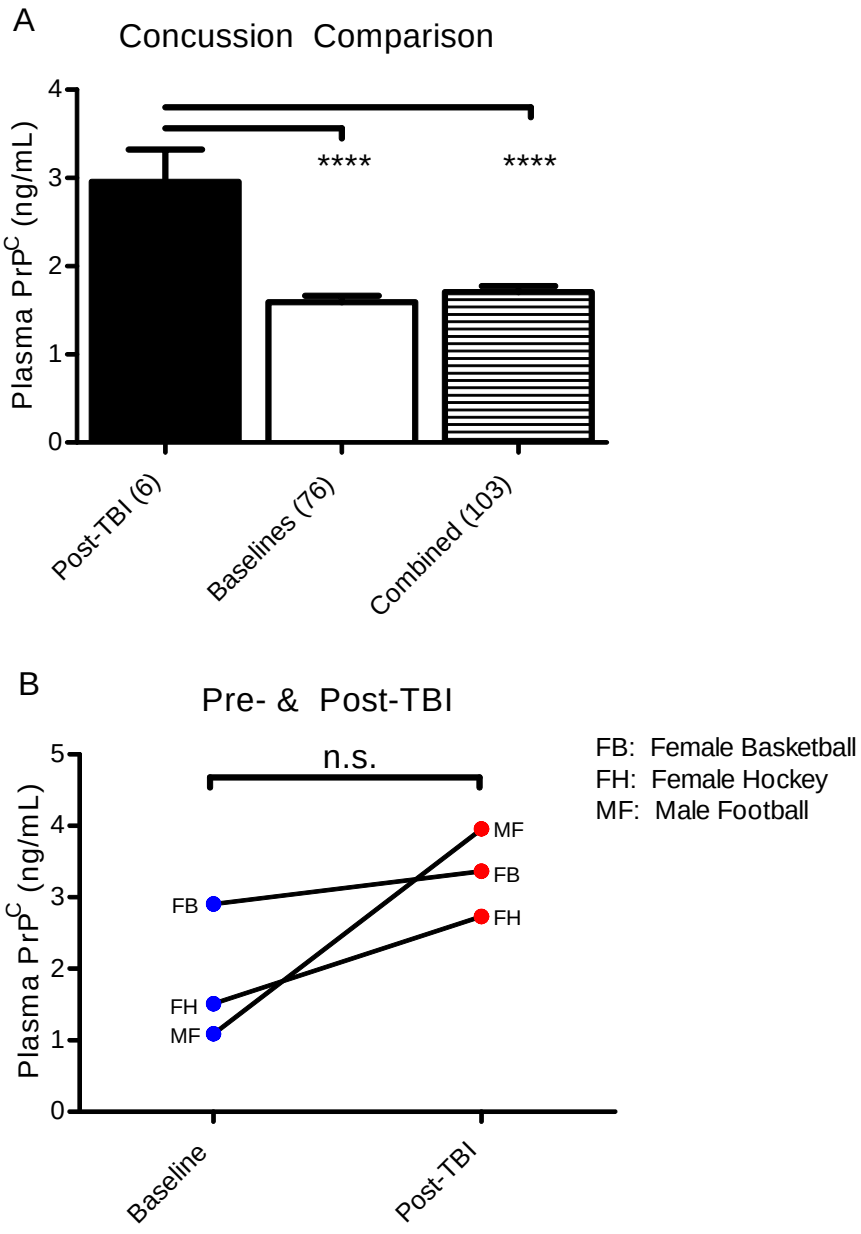
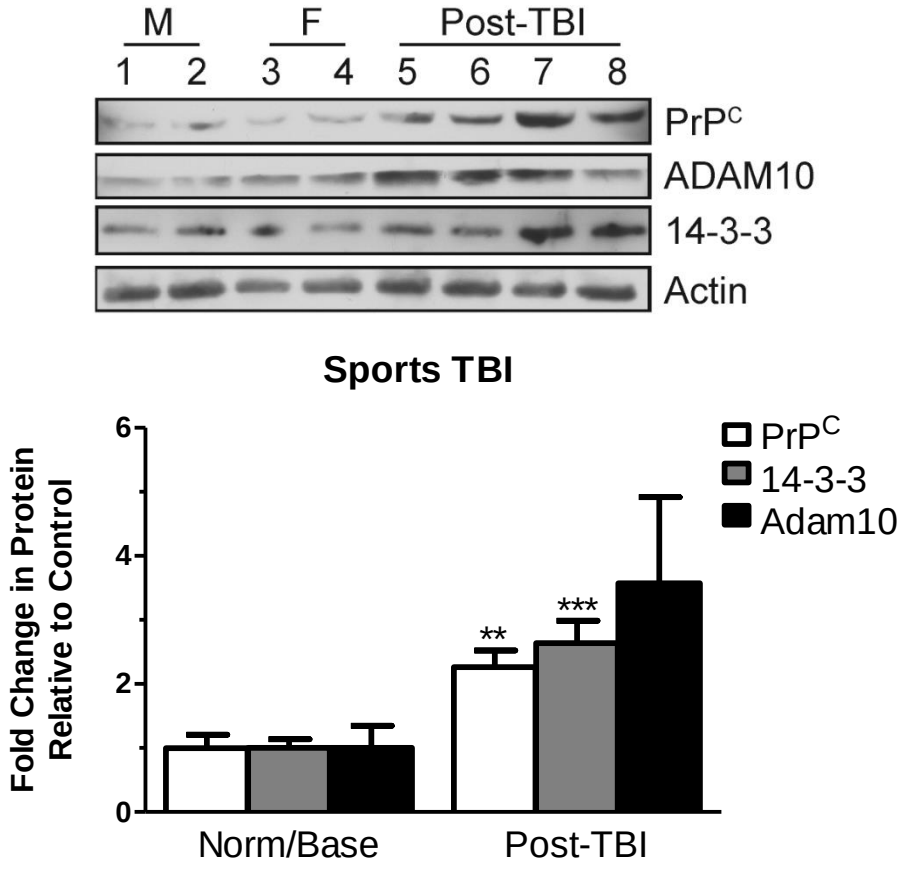


Figure 6.4. Concussed athlete western blotting. T-test comparison of IR for target proteins between baseline/controls (rows 1-4) and post-TBI samples (rows 5-8) was significantly increased for PrP^C ($p<0.01$) and 14-3-3 ($p<0.001$), but was not significant for ADAM10 ($p>0.05$). PrP^C IR is increased in post-TBI samples (n=4, mean 2.26 ± 0.26 fold) compared with baseline/controls (n=8, mean 1.00 ± 0.21 fold). 14-3-3 IR is also increased in post-TBI samples (n=4, mean 2.64 ± 0.36 fold) (n=8, mean 1.00 ± 0.14 fold). ADAM10 IR also showed an increase in post-TBI samples (n=4 mean 3.57 ± 1.34 fold), but was not significant in control/baseline (n=8, mean 1.00 ± 0.35 fold).



Section 7. Discussion

7.1. General Discussion

Traumatic brain injury (TBI) is becoming a growing concern in society. Not only does TBI affect the quality of life for individuals and those close to them, but it also poses legal ramifications with far reaching consequences in the context of sports and the military in regards to improper injury management. The current standards of care all too often fail to properly diagnose those who have suffered a mild TBI, which is the most common injury in those admitted to emergency rooms. Without proper diagnosis, those injured are at risk of developing debilitating symptoms without treatment as well as further injuring themselves that may have catastrophic outcomes. A recent approach towards improving diagnosis of TBI has focused on evaluating protein concentrations in biological fluids that were indicative of injury. In the case of TBI, these protein biomarkers are a result of damaged and degenerating tissue in the central nervous system (CNS) that allows these proteins to be released into the extracellular space. Many proteins have been examined, but the search for feasible protein biomarkers is ongoing as there is no one definitive biomarker. A protein that has been recently reported as a biomarker in various neurodegenerative conditions and diseases is the cellular prion protein (PrP^C). This protein is expressed abundantly in the CNS and is involved in various neuronal functions that collectively suggests it plays a neuroprotective role. Thus begs the theory of whether PrP^C, given its extracellular orientation, in neuronal cells is susceptible to TBI-induced damage that could lead to its release and subsequent pooling within the systemic circulation.

To explore this hypothesis of PrP^C release following TBI, animal studies were conducted using a blast over pressure (BOP) exposure model on Sprague-Dawley (S-D) rats with

collaboration from the Defense Research and Development Canada (DRDC) center in Suffield, Alberta, Canada. Using an advanced blast simulation tube (**see section 4.1**), adult S-D rats received head-only exposure to a single gas-driven primary blast wave at different intensities (15-30 PSI) with different head restraining conditions (restraint, whiplash, or net). 24 hours following exposure, animals were decapitated and exsanguinated for blood samples. Removed brains were subjected to immunohistochemical analysis for cytoarchitecture changes indicative of TBI (**see section 5.2**), while blood samples underwent centrifugation for plasma fraction separation. Plasma samples were analyzed using a modified commercial enzyme-linked immunosorbent assay kit (ELISA) for sensitive quantification of full-length soluble PrP^C. Restraint condition samples were of particular interest because of the blast-only exposure component as opposed to whiplash and net groups, which involved acceleratory/decelerator forces from violent movement of the head and a semi-protective covering respectively. Plasma PrP^C from restraint group animals was indeed increased following blast exposure compared to sham controls (**see section 5.1**). Although interestingly, plasma PrP^C did not strictly increase in a blast intensity-dependent manner as concentrations seemingly taper off and even slightly decrease at higher intensities suggesting an "all-or-nothing" response mechanism. Further study is required at lower blast intensity (<15 PSI) to deduce any mild intensity-dependent changes in plasma PrP^C. Combined analysis of plasma PrP^C from restraint, whiplash, and net groups combined (**see section 5.3.1**) show that PrP^C is significantly elevated following blast in restraint and whiplash groups, while net group results show an increased trend following blast, but was not significant. Similar to restraint group, whiplash results also decreased slightly at higher blast intensity. This may potentially be a result of limited sample size, especially in the case of whiplash. But it may also suggest increased PrP^C uptake in the brain in response to more severe

damage from higher blast intensities. Such a response has been suggested in previous reports in light of PrP^C's involvement in important neuronal functions and neuroprotective qualities (Banks, et al. 2009; Carnini, et al. 2010). The lack of significant increase in plasma PrP^C for net group suggests that the head covering mitigated some of the blast exposure as evident by the slightly elevated levels. Time course profiling of plasma PrP^C over four weeks was also performed for restraint group animals exposed to 25 PSI. PrP^C was significantly elevated after one day, and proceeded to normalize to near control levels by one week, and then returned to control levels by two weeks and beyond (**see section 5.3.2**). These results suggest that PrP^C release remains elevated within the week, likely due to increased expression in the brain in response to secondary injury mechanisms and/or neuroinflammatory mediators. Potential means of prolonged PrP^C release that have previously been associated with TBI include adaptive responses to increased neurotoxicity and oxidative stress, inflammatory CCL2-mediatd release, upregulated ADAM10 sheddase activity, and possibly microvesicular exosome release (Altmeppen, et al. 2011; Brown, et al. 1997b; Cho, et al. 2013; Fevrier, et al. 2004; Graner, et al. 2013; Roberts, et al. 2010; Semple, et al. 2010; Wang, et al. 2012; Warren, et al. 2012). Furthermore, it has previously been reported that following blast exposure *Prnp* is upregulated within the brain, but for however long and to what degree requires further investigation (Kochanek, et al. 2013). Future study is needed to examine the acute release of plasma PrP^C and determine whether there is a peak in PrP^C concentration within 24 hours of blast exposure.

Western blot analysis further verified that PrP^C is increased within the plasma following blast in a manner similar to ELISA results. As previously reported, ADAM10 is upregulated following TBI and can also be secreted into the extracellular space within exosomes where it

actively cleaves cell surface proteins (Stoeck, et al. 2006; Warren, et al. 2012). Combined with increased BBB permeability following TBI, it is possible that soluble ADAM10-containing exosomes are able to cross into the bloodstream. Moreover, ADAM10 may continue to shed surface proteins such as PrP^C that are also packaged during vesicular formation from the plasma membrane. Future study using different region-specific antibodies for PrP^C is needed to determine the degree of fractionation following TBI, which may be indicative of increased ADAM10 activity and/or reactive oxygen species. Another well studied biomarker in TBI is apolipoprotein E (ApoE). ApoE is a plasma lipid carrier produced by astrocytes and is abundant within the brain serving various functions such as neuronal maintenance, repair, growth, and synaptic plasticity (Houlden and Greenwood 2006). Following TBI, ApoE levels within the brain increase as it plays a role in transporting lipids to damaged neurons in aiding repair. Similarly, in a study examining temporal lobe epilepsy, plasma ApoE was shown to dramatically increase compared to controls (Kumar, et al. 2006). Typically, ApoE is investigated in the context of TBI for its ε4 isoform, which is believed to be a risk factor indicator for poor CNS response following TBI (Samatovicz 2000). However, Western blotting was performed using primary antibody specific to a region within the protein's C-terminal allowing binding of all ApoE isoforms as this protein is only polymorphic within its lipid binding N-terminal. Thus, in addition to PrP^C, Western blotting shows ADAM10 and ApoE IR is also increased following blast exposure, more so in restraint group than whiplash. And as expected, IR for markers in net group were not significantly elevated following blast, likely as a result of the protective head covering mitigating imposed damage. It must be noted that for whiplash 15 PSI group, BOP animal experiments show that PrP^C can be used as a marker for bTBI in animals, which begs the question of whether the protein can be used as a marker for mTBI in sports-related concussion.

To address this query, blood plasma was collected from the normal healthy adult population (age 18-30 years old) of the University of Saskatchewan (U of S) student body in addition to concussed student athletes from the U of S Huskies athletic teams for quantification of PrP^C using ELISA. Previous reports of plasma PrP^C concentration are conflicting nor were they sampled from an appropriate age group that could correspond with student athlete participants (Breitling, et al. 2012; Mitsios, et al. 2007; Roberts, et al. 2010; Volkel, et al. 2001). Thus, it was necessary to sample a sufficient number of participants to establish normal values. Controls consisted of non-athlete participants from the U of S in addition to baseline samples collected from athletes during the offseason before regular contact. Significant age and gender variation was not observed in normal plasma PrP^C results, which for our purposes makes PrP^C an ideal biomarker within the general population (**see section 6.1**). Female values were slightly higher than males though not significantly, which is in line with previous reports of a non-significant trend with gender (Breitling, et al. 2012). Interestingly, lower soluble PrP^C concentration was observed among offseason athlete baseline values as compared with non-athlete students. A possible reasoning for this is that collegiate level athletes are well conditioned physically and have lower blood pressure than the general population. Since PrP^C is also expressed on endothelial cell lining (Simak, et al. 2012), those with higher blood pressure would release more PrP^C into the blood as was shown in a hypertensive population (Breitling, et al. 2012). Another example of differing protein levels related to physical fitness was recently reported by Bazarian and coworkers in serum levels of ApoA1 and S100B autoantibody titer between off-season athletes and their controls (Bazarian, et al. 2014). Further study is needed to determine whether plasma PrP^C levels is affected by an individual's fitness level. Analysis of

post-mTBI/concussion samples show a significant rise in plasma PrP^C compared with combined baseline and non-athlete samples (**see section 6.2**). However, comparison of corresponding pre- and post-TBI values did not show significant difference despite an upward trend in the latter. It is noteworthy that of the six post-TBI samples, only three had a paired baseline sample to compare against, and these pairs do show a slight increase in PrP^C concentration despite delayed periods in collection following injury (**see table 6.1**). Extreme examples of this can be seen when comparing the trend for MF and FB samples. The MF post-injury sample was collected shortly after injury (1 day) while the FB was collected much later (6 days) (**see figure 6.3-B**). It is likely that the lack of any appreciable elevation in PrP^C within the FB samples is a result of this time latency. Another factor to consider is that of all the injured athletes sampled, the MF participant had the highest number of self-reported symptoms (21) with high severity (81 out of possible 126 symptom score) (**see table 6.1**). Thus, higher injury severity may contribute towards higher plasma PrP^C following injury in the MF participant. Unfortunately, correlation analysis could not be performed in this study due to non-controlled sampling times following injury, but such considerations would strengthen the feasibility of PrP^C as an ideal biomarker for TBI. Further study is underway and has been broadened to include more serious injuries that are to be collected from patients admitted to the Royal University Hospital (RUH) emergency room.

To further validate that plasma PrP^C can be used as a biomarker for mTBI/concussion, Western blotting was performed for PrP^C and other target proteins that have been previously reported as markers of TBI. 14-3-3 is a CNS-specific intracellular scaffolding protein expressed abundantly on neural and glial cells, and has been reported to be increased in the brain and CSF following mTBI (Arun, et al. 2013; Siman, et al. 2004). Comparison of four post-TBI samples

against combined baseline and normal controls shows there is a significant increase in PrP^C and 14-3-3 (**see figure 6.4**). ADAM10 was also increased but was not significant due to high variability from prolonged collection latency. Further analysis with increased pre- and post-TBI pairings with more acute collection times is required to definitively rule on PrP^C's applicability for mTBI/concussion diagnosis.

mTBI and bTBI are similar in that most cases present with sparse neurodegeneration and injuries are typically not accompanied by complications such as sleep apnea, seizures, or mortality. The identification of surrogate protein biomarkers for determining acute brain damage, secondary brain injury, severity, as well as prognosis remains an active and relevant area of research. Such biomarkers could be used to complement the current standard clinical tests for TBI identification, and facilitate hospital resource allocation and patient management. Sport-related mTBI and military-related bTBI are significant health concerns to athletes and military service-members respectively, as TBI poses a high risk of long-term consequences for individuals who fail to properly manage their injuries. PrP^C is abundantly expressed throughout the CNS. PrP^C's cellular location and functions suggests it has various neuroprotective properties. Previous reports have linked plasma PrP^C levels as biomarkers of brain injury and neurodegeneration, but none have examined its applicability to TBI, which itself involves both acute and prolonged brain injury mechanisms and is highly associated with long-term neurodegenration. The contents of this thesis submit that PrP^C is indeed increased within the plasma following TBI using a BOP animal model and a small human pilot project. If findings from previous reports apply, it is likely that PrP^C is released in the acute period from damaged neurons and is subsequently upregulated in affected areas as a means of ameliorating TBI-mediated dysregulation of signaling pathways and ion homeostasis. Simultaneously, mediators

of secondary injury, upregulated cleavage enzymes, and persistent neurotoxic environment following TBI can contribute towards further release of PrP^C from neurons. Given the protein's ability to traverse the BBB normally, it stands to reason that free PrP^C can accrue even more within the periphery through a highly permeable BBB following TBI. At this time it is not clear whether PrP^C is a highly specific marker of TBI only; further study is required to examine the effects of polytrauma, which includes impact to the head and the body as most cases of TBI are never inclusive of head-only injuries. However, the findings presented here present a promising addition to the large body of evidence comprising the complex pathology of TBI.

7.2. Future Research Goals

- Determine the exact release mechanism of PrP^C following TBI using *in vitro* cell culturing technique. By using antibodies towards ADAM10, we can determine the contribution that ADAM10 may play towards PrP^C cleavage following TBI by comparison with non-treated cultures.
    - Determie if TBI causes fractionation of PrP^C by performing sequential Western blotting with antibodies targeting different epitotes of PrP^C. This is important as different fragments can have distinct physiological effects.

- Determine whether the release of soluble PrP^C following TBI is a neuroprotective phenomenon by using cultured primary neurons isolated from wild type, PrP-KO and Tg20 (overexpresses 20 time more PrP^C) mice in an invitro set up similar to what mentioned above. We can also use recombinant PrP^C in culture media and monitor the outcome with different techniques including immunohistochemical analysis. Furthermore, treatment of PrP^C-knockout cell cultures with various fragments of PrP^C will determine functional regions associated with improved outcome to perhaps provide potential therapeutic targets, such as PrP^C region-mimicking peptides, for acute treatment of TBI. Protein complex immunoprecipitation will be performed on said PrP^C fragments to determine binding complex formation with potential lipid raft receptors or signaling molecules.

- Produce a single and repetive mTBI animal model using a weight drop injury method as previously developed (Marmarou, et al. 1994; Foda, et al., 1994). Sampling of blood

samples during the acute period following injury to delineate whether PrP^C concentrations peak within 24 hours. Also, head-only, body-only, and combined impact trials will be performed to determine whether blows to the body significantly affect plasma PrP^C levels given it's ubiquitous expression.

References

Aguzzi, A., & Calella, A. M. (2009). Prions: protein aggregation and infectious diseases. *Physiol Rev, 89*(4), 1105-1152.

Algattas, H., & Huang, J. H. (2014). Traumatic Brain Injury pathophysiology and treatments: early, intermediate, and late phases post-injury. *Int J Mol Sci, 15*(1), 309-341.

Altmeppen, H. C., Prox, J., Puig, B., Kluth, M. A., Bernreuther, C., Thurm, D., . . . Glatzel, M. (2011). Lack of a-disintegrin-and-metalloproteinase ADAM10 leads to intracellular accumulation and loss of shedding of the cellular prion protein in vivo. *Mol Neurodegener, 6*, 36, 1-12.

Anderson, R. E., Hansson, L. O., Nilsson, O., Dijlai-Merzoug, R., & Settergren, G. (2001). High serum S100B levels for trauma patients without head injuries. *Neurosurgery, 48*(6), 1255-1258.

Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., & Nicotera, P. (1995). Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron, 15*(4), 961-973.

Arun, P., Abu-Taleb, R., Oguntayo, S., Wang, Y., Valiyaveettil, M., Long, J. B., & Nambiar, M. P. (2013). Acute mitochondrial dysfunction after blast exposure: potential role of mitochondrial glutamate oxaloacetate transaminase. *J Neurotrauma, 30*(19), 1645-1651.

Banks, W. A., Robinson, S. M., Diaz-Espinoza, R., Urayama, A., & Soto, C. (2009). Transport of prion protein across the blood-brain barrier. *Exp Neurol, 218*(1), 162-167.

Barkhoudarian, G., Hovda, D. A., & Giza, C. C. (2011). The molecular pathophysiology of concussive brain injury. *Clin Sports Med, 30*(1), 33-48, vii-iii.

Bazarian, J. J., Zhu, T., Zhong, J., Janigro, D., Rozen, E., Roberts, A., . . . Blackman, E. G. (2014). Persistent, Long-term Cerebral White Matter Changes after Sports-Related Repetitive Head Impacts. *PLoS One, 9*(4), e94734.

Belanger, H. G., Vanderploeg, R. D., Curtiss, G., & Warden, D. L. (2007). Recent neuroimaging techniques in mild traumatic brain injury. *J Neuropsychiatry Clin Neurosci, 19*(1), 5-20.

Bendheim, P. E., Bockman, J. M., McKinley, M. P., Kingsbury, D. T., & Prusiner, S. B. (1985). Scrapie and Creutzfeldt-Jakob disease prion proteins share physical properties and antigenic determinants. *Proc Natl Acad Sci U S A, 82*(4), 997-1001.

Bendheim, P. E., Brown, H. R., Rudelli, R. D., Scala, L. J., Goller, N. L., Wen, G. Y., . . . Bolton, D. C. (1992). Nearly Ubiquitous Tissue Distribution of the Scrapie Agent Precursor Protein. *Neurology, 42*(1), 149-156.

Beraldo, F. H., Arantes, C. P., Santos, T. G., Machado, C. F., Roffe, M., Hajj, G. N., . . . Martins, V. R. (2011). Metabotropic glutamate receptors transduce signals for neurite outgrowth after binding of the prion protein to laminin gamma1 chain. *FASEB J, 25*(1), 265-279.

Boden, B. P., Tacchetti, R. L., Cantu, R. C., Knowles, S. B., & Mueller, F. O. (2007). Catastrophic head injuries in high school and college football players. *Am J Sports Med, 35*(7), 1075-1081.

Breitling, L. P., Muller, H., Stegmaier, C., Kliegel, M., & Brenner, H. (2012). Association of prion protein with cognitive functioning in humans. *Experimental Gerontology, 47*(12), 919-924.

Bremer, J., Baumann, F., Tiberi, C., Wessig, C., Fischer, H., Schwarz, P., . . . Aguzzi, A. (2010). Axonal prion protein is required for peripheral myelin maintenance. *Nat Neurosci, 13*(3), 310-318.

Brown, D. R. (2001). Prion and prejudice: normal protein and the synapse. *Trends in Neurosciences, 24*(2), 85-90.

Brown, D. R., & Mohn, C. M. (1999). Astrocytic glutamate uptake and prion protein expression. *Glia, 25*(3), 282-292.

Brown, D. R., Qin, K. F., Herms, J. W., Madlung, A., Manson, J., Strome, R., . . . Kretzschmar, H. (1997). The cellular prion protein binds copper in vivo. *Nature, 390*(6661), 684-687.

Brown, D. R., Schmidt, B., & Kretzschmar, H. A. (1998). Effects of copper on survival of prion protein knockout neurons and glia. *J Neurochem, 70*(4), 1686-1693.

Brown, D. R., Schulz-Schaeffer, W. J., Schmidt, B., & Kretzschmar, H. A. (1997). Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp Neurol, 146*(1), 104-112.

Brown, D. R., Wong, B. S., Hafiz, F., Clive, C., Haswell, S. J., & Jones, I. M. (1999). Normal prion protein has an activity like that of superoxide dismutase. *Biochem J, 344 Pt 1*, 1-5.

Brown, P., Rohwer, R. G., Dunstan, B. C., MacAuley, C., Gajdusek, D. C., & Drohan, W. N. (1998). The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. *Transfusion, 38*(9), 810-816.

Bueler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H. P., DeArmond, S. J., . . . Weissmann, C. (1992). Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature, 356*(6370), 577-582.

Burbach, G. J., Dehn, D., Del Turco, D., Staufenbiel, M., & Deller, T. (2004). Laser microdissection reveals regional and cellular differences in GFAP mRNA upregulation

following brain injury, axonal denervation, and amyloid plaque deposition. *Glia, 48*(1), 76-84.

Caby, M. P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G., & Bonnerot, C. (2005). Exosomal-like vesicles are present in human blood plasma. *Int Immunol, 17*(7), 879-887.

Carnini, A., Casha, S., Yong, V. W., Hurlbert, R. J., & Braun, J. E. (2010). Reduction of PrP(C) in human cerebrospinal fluid after spinal cord injury. *Prion, 4*(2), 80-86.

Carulla, P., Bribian, A., Rangel, A., Gavin, R., Ferrer, I., Caelles, C., . . . Llorens, F. (2011). Neuroprotective role of PrPC against kainate-induced epileptic seizures and cell death depends on the modulation of JNK3 activation by GluR6/7-PSD-95 binding. *Mol Biol Cell, 22*(17), 3041-3054.

Cashman, N. R., Loertscher, R., Nalbantoglu, J., Shaw, I., Kascsak, R. J., Bolton, D. C., & Bendheim, P. E. (1990). Cellular isoform of the scrapie agent protein participates in lymphocyte activation. *Cell, 61*(1), 185-192.

Cernak, I., Wang, Z., Jiang, J., Bian, X., & Savic, J. (2001). Ultrastructural and functional characteristics of blast injury-induced neurotrauma. *J Trauma, 50*(4), 695-706.

Chen, Y., Huang, W., & Constantini, S. (2013). The Differences between Blast-Induced and Sports-Related Brain Injuries. *Front Neurol, 4*, 119.

Cho, H. J., Sajja, V. S., Vandevord, P. J., & Lee, Y. W. (2013). Blast induces oxidative stress, inflammation, neuronal loss and subsequent short-term memory impairment in rats. *Neuroscience, 253*, 9-20.

Choi, C. J., Anantharam, V., Saetveit, N. J., Houk, R. S., Kanthasamy, A., & Kanthasamy, A. G. (2007). Normal cellular prion protein protects against manganese-induced oxidative stress and apoptotic cell death. *Toxicol Sci, 98*(2), 495-509.

Clark, R. S., Kochanek, P. M., Watkins, S. C., Chen, M., Dixon, C. E., Seidberg, N. A., . . . Graham, S. H. (2000). Caspase-3 mediated neuronal death after traumatic brain injury in rats. *J Neurochem, 74*(2), 740-753.

Colling, S. B., Collinge, J., & Jefferys, J. G. (1996). Hippocampal slices from prion protein null mice: disrupted Ca(2+)-activated K+ currents. *Neurosci Lett, 209*(1), 49-52.

Colling, S. B., Khana, M., Collinge, J., & Jefferys, J. G. (1997). Mossy fibre reorganization in the hippocampus of prion protein null mice. *Brain Res, 755*(1), 28-35.

Collinge, J., Whittington, M. A., Sidle, K. C. L., Smith, C. J., Palmer, M. S., Clarke, A. R., & Jefferys, J. G. R. (1994). Prion Protein Is Necessary for Normal Synaptic Function. *Nature, 370*(6487), 295-297.

Daneshvar, D. H., Nowinski, C. J., McKee, A. C., & Cantu, R. C. (2011). The epidemiology of sport-related concussion. *Clin Sports Med, 30*(1), 1-17, vii.

De Gasperi, R., Gama Sosa, M. A., Kim, S. H., Steele, J. W., Shaughness, M. C., Maudlin-Jeronimo, E., . . . Elder, G. A. (2012). Acute blast injury reduces brain abeta in two rodent species. *Front Neurol, 3*, 177.

de Lanerolle, N. C., Bandak, F., Kang, D., Li, A. Y., Du, F., Swauger, P., . . . Kim, J. H. (2011). Characteristics of an explosive blast-induced brain injury in an experimental model. *J Neuropathol Exp Neurol, 70*(11), 1046-1057.

Desai, V., & Kaler, S. G. (2008). Role of copper in human neurological disorders. *Am J Clin Nutr, 88*(3), 855S-858S.

Dodelet, V. C., & Cashman, N. R. (1998). Prion protein expression in human leukocyte differentiation. *Blood, 91*(5), 1556-1561.

Effgen, G. B., Vogel, E. W., 3rd, Lynch, K. A., Lobel, A., Hue, C. D., Meaney, D. F., . . . Morrison, B., 3rd. (2014). Isolated primary blast alters neuronal function with minimal cell death in organotypic hippocampal slice cultures. *J Neurotrauma, 31*(13), 1202-1210.

Fevrier, B., Vilette, D., Archer, F., Loew, D., Faigle, W., Vidal, M., . . . Raposo, G. (2004). Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A, 101*(26), 9683-9688.

Fluharty, B. R., Biasini, E., Stravalaci, M., Sclip, A., Diomede, L., Balducci, C., . . . Harris, D. A. (2013). An N-terminal fragment of the prion protein binds to amyloid-beta oligomers and inhibits their neurotoxicity in vivo. *J Biol Chem, 288*(11), 7857-7866.

Foda, M. A., & Marmarou, A. (1994). A new model of diffuse brain injury in rats. Part II: Morphological characterization. *J Neurosurg, 80*(2), 301-313.

Fournier, J. G., Escaig-Haye, F., & Grigoriev, V. (2000). Ultrastructural localization of prion proteins: physiological and pathological implications. *Microsc Res Tech, 50*(1), 76-88.

Fritz, H. G., Walter, B., Holzmayr, M., Brodhun, M., Patt, S., & Bauer, R. (2005). A pig model with secondary increase of intracranial pressure after severe traumatic brain injury and temporary blood loss. *J Neurotrauma, 22*(7), 807-821.

Gavett, B. E., Stern, R. A., & McKee, A. C. (2011). Chronic traumatic encephalopathy: a potential late effect of sport-related concussive and subconcussive head trauma. *Clin Sports Med, 30*(1), 179-188, xi.

Goldstein, L. E., Fisher, A. M., Tagge, C. A., Zhang, X. L., Velisek, L., Sullivan, J. A., . . . McKee, A. C. (2012). Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model. *Sci Transl Med, 4*(134), 134ra160.

Graner, M. W., Epple, L. M., Dusto, N. L., Lencioni, A. M., Nega, M., Herring, M., . . . Anchordoquy, T. J. (2013). Circulating exosomes as new biomarkers for brain disease and injury. *Sensing Technologies for Global Health, Military Medicine, and Environmental Monitoring Iii, 8723*.

Gupta, A., & Pulliam, L. (2014). Exosomes as mediators of neuroinflammation. *J Neuroinflammation, 11*, 68.

Guskiewicz, K. M., Register-Mihalik, J., McCrory, P., McCrea, M., Johnston, K., Makdissi, M., . . . Meeuwisse, W. (2013). Evidence-based approach to revising the SCAT2: introducing the SCAT3. *Br J Sports Med, 47*(5), 289-293.

Haeberle, A. M., Ribaut-Barassin, C., Bombarde, G., Mariani, J., Hunsmann, G., Grassi, J., & Bailly, Y. (2000). Synaptic prion protein immuno-reactivity in the rodent cerebellum. *Microsc Res Tech, 50*(1), 66-75.

Halliday, S., Houston, F., & Hunter, N. (2005). Expression of PrPC on cellular components of sheep blood. *J Gen Virol, 86*(Pt 5), 1571-1579.

Halstead, M. E., & Walter, K. D. (2010). American Academy of Pediatrics. Clinical report--sport-related concussion in children and adolescents. *Pediatrics, 126*(3), 597-615.

Harmon, K. G., Drezner, J., Gammons, M., Guskiewicz, K., Halstead, M., Herring, S., . . . Roberts, W. (2013). American Medical Society for Sports Medicine position statement: concussion in sport. *Clin J Sport Med, 23*(1), 1-18.

Herms, J., Tings, T., Gall, S., Madlung, A., Giese, A., Siebert, H., . . . Kretzschmar, H. (1999). Evidence of presynaptic location and function of the prion protein. *J Neurosci, 19*(20), 8866-8875.

Homsi, S., Federico, F., Croci, N., Palmier, B., Plotkine, M., Marchand-Leroux, C., & Jafarian-Tehrani, M. (2009). Minocycline effects on cerebral edema: relations with inflammatory and oxidative stress markers following traumatic brain injury in mice. *Brain Res, 1291*, 122-132.

Hooper, N. M., Taylor, D. R., & Watt, N. T. (2008). Mechanism of the metal-mediated endocytosis of the prion protein. *Biochem Soc Trans, 36*(Pt 6), 1272-1276.

Hoshino, S., Inoue, K., Yokoyama, T., Kobayashi, S., Asakura, T., Teramoto, A., & Itohara, S. (2003). Prions prevent brain damage after experimental brain injury: a preliminary report. *Acta Neurochir Suppl, 86*, 297-299.

Houlden, H., & Greenwood, R. (2006). Apolipoprotein E4 and traumatic brain injury. *J Neurol Neurosurg Psychiatry, 77*(10), 1106-1107.

Houston, F., Foster, J. D., Chong, A., Hunter, N., & Bostock, C. J. (2000). Transmission of BSE by blood transfusion in sheep. *Lancet, 356*(9234), 999-1000.

Houston, F., McCutcheon, S., Goldmann, W., Chong, A., Foster, J., Siso, S., . . . Hunter, N. (2008). Prion diseases are efficiently transmitted by blood transfusion in sheep. *Blood, 112*(12), 4739-4745.

Hu, B., Liu, C., Bramlett, H., Sick, T. J., Alonso, O. F., Chen, S., & Dietrich, W. D. (2004). Changes in trkB-ERK1/2-CREB/Elk-1 pathways in hippocampal mossy fiber organization after traumatic brain injury. *J Cereb Blood Flow Metab, 24*(8), 934-943.

Jackson, G. S., Murray, I., Hosszu, L. L., Gibbs, N., Waltho, J. P., Clarke, A. R., & Collinge, J. (2001). Location and properties of metal-binding sites on the human prion protein. *Proc Natl Acad Sci U S A, 98*(15), 8531-8535.

Jeter, C. B., Hergenroeder, G. W., Hylin, M. J., Redell, J. B., Moore, A. N., & Dash, P. K. (2013). Biomarkers for the diagnosis and prognosis of mild traumatic brain injury/concussion. *J Neurotrauma, 30*(8), 657-670.

Johnson, V. E., Stewart, W., & Smith, D. H. (2012). Widespread tau and amyloid-beta pathology many years after a single traumatic brain injury in humans. *Brain Pathol, 22*(2), 142-149.

Johnson, C. J., Gilbert, P. U., Abrecht, M., Baldwin, K. L., Russell, R.E., Pedersen, J. A., Aiken, J. M., McKenzie, D. (2013). Low copper and high manganese levels in prion protein plaques. *Viruses 5*(2), 654-662.

Kamnaksh, A., Kwon, S. K., Kovesdi, E., Ahmed, F., Barry, E. S., Grunberg, N. E., . . . Agoston, D. (2012). Neurobehavioral, cellular, and molecular consequences of single and multiple mild blast exposure. *Electrophoresis, 33*(24), 3680-3692.

Kane, M. J., Angoa-Perez, M., Briggs, D. I., Viano, D. C., Kreipke, C. W., & Kuhn, D. M. (2012). A mouse model of human repetitive mild traumatic brain injury. *J Neurosci Methods, 203*(1), 41-49.

Keller, S., Sanderson, M. P., Stoeck, A., & Altevogt, P. (2006). Exosomes: from biogenesis and secretion to biological function. *Immunol Lett, 107*(2), 102-108.

Khosravani, H., Zhang, Y., Tsutsui, S., Hameed, S., Altier, C., Hamid, J., . . . Zamponi, G. W. (2008). Prion protein attenuates excitotoxicity by inhibiting NMDA receptors. *J Cell Biol, 181*(3), 551-565.

Kleene, R., Loers, G., Langer, J., Frobert, Y., Buck, F., & Schachner, M. (2007). Prion protein regulates glutamate-dependent lactate transport of astrocytes. *J Neurosci, 27*(45), 12331-12340.

Kochanek, P. M., Dixon, C. E., Shellington, D. K., Shin, S. S., Bayir, H., Jackson, E. K., . . . Jenkins, L. W. (2013). Screening of biochemical and molecular mechanisms of secondary injury and repair in the brain after experimental blast-induced traumatic brain injury in rats. *J Neurotrauma, 30*(11), 920-937.

Krupinski, J., Turu, M. M., Luque, A., Badimon, L., & Slevin, M. (2008). Increased PrPC expression correlates with endoglin (CD105) positive microvessels in advanced carotid lesions. *Acta Neuropathol, 116*(5), 537-545.

Kumar, A., Tripathi, M., Pandey, R. M., Ramakrishnan, L., Srinivas, M., Luthra, K. (2006). Apolipoprotein E in temporal lobe epilepsy: A case-control study. *Disease markeres, 22*(5/6), 335-342.

Kuwahara, C., Takeuchi, A. M., Nishimura, T., Haraguchi, K., Kubosaki, A., Matsumoto, Y., . . . Onodera, T. (1999). Prions prevent neuronal cell-line death. *Nature, 400*(6741), 225-226.

Langlois, J. A., Rutland-Brown, W., & Wald, M. M. (2006). The epidemiology and impact of traumatic brain injury: a brief overview. *J Head Trauma Rehabil, 21*(5), 375-378.

Lauren, J., Gimbel, D. A., Nygaard, H. B., Gilbert, J. W., & Strittmatter, S. M. (2009). Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature, 457*(7233), 1128-1132.

Liu, J., Liu, M. C., & Wang, K. K. (2008). Calpain in the CNS: from synaptic function to neurotoxicity. *Sci Signal, 1*(14), re1.

Liu, L., Jiang, D., McDonald, A., Hao, Y., Millhauser, G. L., & Zhou, F. (2011). Copper redox cycling in the prion protein depends critically on binding mode. *J Am Chem Soc, 133*(31), 12229-12237.

Loane, D. J., Pocivavsek, A., Moussa, C. E., Thompson, R., Matsuoka, Y., Faden, A. I., . . . Burns, M. P. (2009). Amyloid precursor protein secretases as therapeutic targets for traumatic brain injury. *Nat Med, 15*(4), 377-379.

MacGregor, I., Hope, J., Barnard, G., Kirby, L., Drummond, O., Pepper, D., . . . Prowse, C. (1999). Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. *Vox Sang, 77*(2), 88-96.

Manson, J. C., Clarke, A. R., Hooper, M. L., Aitchison, L., McConnell, I., & Hope, J. (1994). 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. *Mol Neurobiol, 8*(2-3), 121-127.

Marchi, N., Bazarian, J. J., Puvenna, V., Janigro, M., Ghosh, C., Zhong, J., . . . Janigro, D. (2013). Consequences of repeated blood-brain barrier disruption in football players. *PLoS One, 8*(3), e56805.

Marmarou, A., Foda, M. A., van den Brink, W., Campbell, J., Kita, H., & Demetriadou, K. (1994). A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. *J Neurosurg, 80*(2), 291-300.

Mayer, M. L. (2005). Glutamate receptor ion channels. *Curr Opin Neurobiol, 15*(3), 282-288.

Mayorga, M. A. (1997). The pathology of primary blast overpressure injury. *Toxicology, 121*(1), 17-28.

McKee, A. C., Cantu, R. C., Nowinski, C. J., Hedley-Whyte, E. T., Gavett, B. E., Budson, A. E., . . . Stern, R. A. (2009). Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. *J Neuropathol Exp Neurol, 68*(7), 709-735.

McKee, A. C., Daneshvar, D. H., Alvarez, V. E., & Stein, T. D. (2014). The neuropathology of sport. *Acta Neuropathol, 127*(1), 29-51.

McKee, A. C., & Robinson, M. E. (2014). Military-related traumatic brain injury and neurodegeneration. *Alzheimers Dement, 10*(3 Suppl), S242-253.

McLennan, N. F., Brennan, P. M., McNeill, A., Davies, I., Fotheringham, A., Rennison, K. A., . . . Bell, J. E. (2004). Prion protein accumulation and neuroprotection in hypoxic brain damage. *American Journal of Pathology, 165*(1), 227-235.

Meehan, W. P., 3rd, Mannix, R. C., O'Brien, M. J., & Collins, M. W. (2013). The prevalence of undiagnosed concussions in athletes. *Clin J Sport Med, 23*(5), 339-342.

Meehan, W. P., 3rd, & Micheli, L. J. (2011). Concussion results in deficits in neurocognitive functioning. Preface. *Clin Sports Med, 30*(1), xvii-iii.

Megra, B., Eugenin, E., Roberts, T., Morgello, S., & Berman, J. W. (2013). Protease resistant protein cellular isoform (PrP(c)) as a biomarker: clues into the pathogenesis of HAND. *J Neuroimmune Pharmacol, 8*(5), 1159-1166.

Metting, Z., Wilczak, N., Rodiger, L. A., Schaaf, J. M., & van der Naalt, J. (2012). GFAP and S100B in the acute phase of mild traumatic brain injury. *Neurology, 78*, 1428-1433.

Meyne, F., Gloeckner, S. F., Ciesielczyk, B., Heinemann, U., Krasnianski, A., Meissner, B., & Zerr, I. (2009). Total prion protein levels in the cerebrospinal fluid are reduced in patients with various neurological disorders. *Journal of Alzheimers Disease, 17*(4), 863-873.

Mitsios, N., Saka, M., Krupinski, J., Pennucci, R., Sanfeliu, C., Miguel Turu, M., . . . Slevin, M. (2007). Cellular prion protein is increased in the plasma and peri-infarcted brain tissue after acute stroke. *J Neurosci Res, 85*(3), 602-611.

Moser, M., Colello, R. J., Pott, U., & Oesch, B. (1995). Developmental expression of the prion protein gene in glial cells. *Neuron, 14*(3), 509-517.

Nakagawa, A., Manley, G. T., Gean, A. D., Ohtani, K., Armonda, R., Tsukamoto, A., …Tominaga, T. (2011). Mechanisms of primary blast-induced traumatic brain injury: insights from shock-wave research. *J. Neurotrauma,* 28, 1101–1119

Neselius, S., Brisby, H., Theodorsson, A., Blennow, K., Zetterberg, H., & Marcusson, J. (2012). CSF-biomarkers in Olympic boxing: diagnosis and effects of repetitive head trauma. *PLoS One, 7*(4), e33606.

Nunziante, M., Gilch, S., & Schatzl, H. M. (2003). Essential role of the prion protein N terminus in subcellular trafficking and half-life of cellular prion protein. *J Biol Chem, 278*(6), 3726-3734.

Oesch, B., Westaway, D., Walchli, M., McKinley, M. P., Kent, S. B., Aebersold, R., . . . et al. (1985). A cellular gene encodes scrapie PrP 27-30 protein. *Cell, 40*(4), 735-746.

Omalu, B. I., DeKosky, S. T., Minster, R. L., Kamboh, M. I., Hamilton, R. L., & Wecht, C. H. (2005). Chronic traumatic encephalopathy in a National Football League player. *Neurosurgery, 57*(1), 128-134; discussion 128-134.

Paitel, E., Alves da Costa, C., Vilette, D., Grassi, J., & Checler, F. (2002). Overexpression of PrPc triggers caspase 3 activation: potentiation by proteasome inhibitors and blockade by anti-PrP antibodies. *J Neurochem, 83*(5), 1208-1214.

Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., . . . Prusiner, S. B. (1993). Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A, 90*(23), 10962-10966.

Panigaj, M., Brouckova, A., Glierova, H., Dvorakova, E., Simak, J., Vostal, J. G., & Holada, K. (2011). Underestimation of the expression of cellular prion protein on human red blood cells. *Transfusion, 51*(5), 1012-1021.

Parkyn, C. J., Vermeulen, E. G., Mootoosamy, R. C., Sunyach, C., Jacobsen, C., Oxvig, C., . . . Morris, R. J. (2008). LRP1 controls biosynthetic and endocytic trafficking of neuronal prion protein. *J Cell Sci, 121*(Pt 6), 773-783.

Parsons, M. P., & Raymond, L. A. (2014). Extrasynaptic NMDA receptor involvement in central nervous system disorders. *Neuron, 82*(2), 279-293.

Patel, D. R., & Reddy, V. (2010). Sport-related concussion in adolescents. *Pediatr Clin North Am, 57*(3), 649-670.

Pauly, P. C., & Harris, D. A. (1998). Copper stimulates endocytosis of the prion protein. *J Biol Chem, 273*(50), 33107-33110.

Perera, W. S., & Hooper, N. M. (2001). Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region. *Curr Biol, 11*(7), 519-523.

Perini, F., Frangione, B., & Prelli, F. (1996). Prion protein released by platelets. *Lancet, 347*(9015), 1635-1636.

Pham, N., Dhar, A., Khalaj, S., Desai, K., & Taghibiglou, C. (2014). Down regulation of brain cellular prion protein in an animal model of insulin resistance: possible implication in increased prevalence of stroke in pre-diabetics/diabetics. *Biochem Biophys Res Commun, 448*(2), 151-156.

Picard-Hagen, N., Gayrard, V., Viguie, C., Moudjou, M., Imbs, C., & Toutain, P. L. (2006). Prion protein in the cerebrospinal fluid of healthy and naturally scrapie-affected sheep. *J Gen Virol, 87*(Pt 12), 3723-3727.

Plum, L. M., Rink, L., & Haase, H. (2010). The essential toxin: impact of zinc on human health. *Int J Environ Res Public Health, 7*(4), 1342-1365.

Prusiner, S. B. (1982). Novel proteinaceous infectious particles cause scrapie. *Science, 216*(4542), 136-144.

Prusiner, S. B. (1986). Prions are novel infectious pathogens causing scrapie and Creutzfeldt-Jakob disease. *Bioessays, 5*(6), 281-286.

Pushie, M. J., Pickering, I. J., Martin, G. R., Tsutsui, S., Jirik, F. R., & George, G. N. (2011). Prion protein expression level alters regional copper, iron and zinc content in the mouse brain. *Metallomics, 3*(2), 206-214.

Redell, J. B., Moore, A. N., Ward, N. H., 3rd, Hergenroeder, G. W., & Dash, P. K. (2010). Human traumatic brain injury alters plasma microRNA levels. *J Neurotrauma, 27*(12), 2147-2156.

Ritchie, A. J., Crawford, D. M., Ferguson, D. J., Burthem, J., & Roberts, D. J. (2013). Normal prion protein is expressed on exosomes isolated from human plasma. *Br J Haematol, 163*(5), 678-680.

Roberts, T. K., Eugenin, E. A., Morgello, S., Clements, J. E., Zink, M. C., & Berman, J. W. (2010). PrPC, the cellular isoform of the human prion protein, is a novel biomarker of HIV-associated neurocognitive impairment and mediates neuroinflammation. *Am J Pathol, 177*(4), 1848-1860.

Sales, N., Rodolfo, K., Hassig, R., Faucheux, B., Di Giamberardino, L., & Moya, K. L. (1998). Cellular prion protein localization in rodent and primate brain. *Eur J Neurosci, 10*(7), 2464-2471.

Samatovicz, R. A. (2000). Genetics and brain injury: apolipoprotein E. *J Head Trauma Rehabil, 15*(3), 869-874.

Semple, B. D., Bye, N., Rancan, M., Ziebell, J. M., & Morganti-Kossmann, M. C. (2010). Role of CCL2 (MCP-1) in traumatic brain injury (TBI): evidence from severe TBI patients and CCL2-/- mice. *J Cereb Blood Flow Metab, 30*(4), 769-782.

Sensi, S. L., Paoletti, P., Bush, A. I., & Sekler, I. (2009). Zinc in the physiology and pathology of the CNS. *Nat Rev Neurosci, 10*(11), 780-791.

Shahim, P., Tegner, Y., Wilson, D. H., Randall, J., Skillback, T., Pazooki, D., . . . Zetterberg, H. (2014). Blood Biomarkers for Brain Injury in Concussed Professional Ice Hockey Players. *JAMA Neurol*.

Sharma, P., Schiapparelli, L., & Cline, H. T. (2013). Exosomes function in cell-cell communication during brain circuit development. *Curr Opin Neurobiol, 23*(6), 997-1004.

Shaw, G., Yang, C., Ellis, R., Anderson, K., Parker Mickle, J., Scheff, S., . . . Howland, D. R. (2005). Hyperphosphorylated neurofilament NF-H is a serum biomarker of axonal injury. *Biochem Biophys Res Commun, 336*(4), 1268-1277.

Shyng, S. L., Heuser, J. E., & Harris, D. A. (1994). A Glycolipid-Anchored Prion Protein Is Endocytosed Via Clathrin-Coated Pits. *Journal of Cell Biology, 125*(6), 1239-1250.

Shyng, S. L., Huber, M. T., & Harris, D. A. (1993). A Prion Protein Cycles between the Cell-Surface and an Endocytic Compartment in Cultured Neuroblastoma-Cells. *Journal of Biological Chemistry, 268*(21), 15922-15928.

Shyu, W. C., Lin, S. Z., Chiang, M. F., Ding, D. C., Li, K. W., Chen, S. F., . . . Li, H. (2005). Overexpression of PrPC by adenovirus-mediated gene targeting reduces ischemic injury in a stroke rat model. *J Neurosci, 25*(39), 8967-8977.

Simak, J., Holada, K., D'Agnillo, F., Janota, J., & Vostal, J. G. (2002). Cellular prion protein is expressed on endothelial cells and is released during apoptosis on membrane microparticles found in human plasma. *Transfusion, 42*(3), 334-342.

Siman, R., McIntosh, T. K., Soltesz, K. M., Chen, Z., Neumar, R. W., & Roberts, V. L. (2004). Proteins released from degenerating neurons are surrogate markers for acute brain damage. *Neurobiol Dis, 16*(2), 311-320.

Siman, R., Toraskar, N., Dang, A., McNeil, E., McGarvey, M. . . . Grady, M. S. (2009). A panel of neuron-enriched proteins as markers for traumatic brain injury in humans. *J Neurotrauma 26*(11), 1867-1877

Singh, N., Das, D., Singh, A., & Mohan, M. L. (2010). Prion protein and metal interaction: physiological and pathological implications. *Curr Issues Mol Biol, 12*(2), 99-107.

Smith, D. H., Chen, X. H., Iwata, A., & Graham, D. I. (2003). Amyloid beta accumulation in axons after traumatic brain injury in humans. *J Neurosurg, 98*(5), 1072-1077.

Spudich, A., Frigg, R., Kilic, E., Kilic, U., Oesch, B., Raeber, A., . . . Hermann, D. M. (2005). Aggravation of ischemic brain injury by prion protein deficiency: role of ERK-1/-2 and STAT-1. *Neurobiol Dis, 20*(2), 442-449.

Stahl, N., Baldwin, M. A., Hecker, R., Pan, K. M., Burlingame, A. L., & Prusiner, S. B. (1992). Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. *Biochemistry, 31*(21), 5043-5053.

Starke, R., Drummond, O., MacGregor, I., Biggerstaff, J., Gale, R., Camilleri, R., . . . Harrison, P. (2002). The expression of prion protein by endothelial cells: a source of the plasma form of prion protein? *Br J Haematol, 119*(3), 863-873.

Stewart, R. S., Drisaldi, B., & Harris, D. A. (2001). A transmembrane form of the prion protein contains an uncleaved signal peptide and is retained in the endoplasmic Reticulum. *Mol Biol Cell, 12*(4), 881-889.

Stoeck, A., Keller, S., Riedle, S., Sanderson, M. P., Runz, S., Le Naour, F., . . . Altevogt, P. (2006). A role for exosomes in the constitutive and stimulus-induced ectodomain cleavage of L1 and CD44. *Biochem J, 393*(Pt 3), 609-618.

Strathmann, F. G., Schulte, S., Goerl, K., & Petron, D. J. (2014). Blood-based biomarkers for traumatic brain injury: Evaluation of research approaches, available methods and potential utility from the clinician and clinical laboratory perspectives. *Clin Biochem., 47*(10-11), 876-888

Sumudhu, W., Perera, S., & Hooper, N. M. (2001). Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region. *Current Biology, 11*(7), 519-523.

Sunyach, C., Jen, A., Deng, J., Fitzgerald, K. T., Frobert, Y., Grassi, J., . . . Morris, R. (2003). The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein. *EMBO J, 22*(14), 3591-3601.

Szewczyk, B. (2013). Zinc homeostasis and neurodegenerative disorders. *Front Aging Neurosci, 5*, 33, 1-12.

Takagi, Y., Harada, J., Chiarugi, A., & Moskowitz, M. A. (2002). STAT1 is activated in neurons after ischemia and contributes to ischemic brain injury. *J Cereb Blood Flow Metab, 22*(11), 1311-1318.

Taraboulos, A., Rogers, M., Borchelt, D. R., McKinley, M. P., Scott, M., Serban, D., & Prusiner, S. B. (1990). Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation. *Proc Natl Acad Sci U S A, 87*(21), 8262-8266.

Tobler, I., Gaus, S. E., Deboer, T., Achermann, P., Fischer, M., Rulicke, T., . . . Manson, J. C. (1996). Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature, 380*(6575), 639-642.

Tsutsui, S., Hahn, J. N., Johnson, T. A., Ali, Z., & Jirik, F. R. (2008). Absence of the cellular prion protein exacerbates and prolongs neuroinflammation in experimental autoimmune encephalomyelitis. *Am J Pathol, 173*(4), 1029-1041.

Um, J. W., Kaufman, A. C., Kostylev, M., Heiss, J. K., Stagi, M., Takahashi, H., . . . Strittmatter, S. M. (2013). Metabotropic glutamate receptor 5 is a coreceptor for Alzheimer abeta oligomer bound to cellular prion protein. *Neuron, 79*(5), 887-902.

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., & Lotvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol, 9*(6), 654-659.

Viegas, P., Chaverot, N., Enslen, H., Perriere, N., Couraud, P. O., & Cazaubon, S. (2006). Junctional expression of the prion protein PrPC by brain endothelial cells: a role in trans-endothelial migration of human monocytes. *J Cell Sci, 119*(Pt 22), 4634-4643.

Vilches, S., Vergara, C., Nicolas, O., Sanclimens, G., Merino, S., Varon, S., . . . Gavin, R. (2013). Neurotoxicity of prion peptides mimicking the central domain of the cellular prion protein. *PLoS One, 8*(8), e70881.

Volkel, D., Zimmermann, K., Zerr, I., Bodemer, M., Lindner, T., Turecek, P. L., . . . Schwarz, H. P. (2001). Immunochemical determination of cellular prion protein in plasma from healthy subjects and patients with sporadic CJD or other neurologic diseases. *Transfusion, 41*(4), 441-448.

Vos, P. E., Jacobs, B., Andriessen, T. M., Lamers, K. J., Borm, G. F., Beems, T., . . . Vissers, J. L. (2010). GFAP and S100B are biomarkers of traumatic brain injury: an observational cohort study. *Neurology, 75*, 1786-1793.

Vostal, J. G., Holada, K., & Simak, J. (2001). Expression of cellular prion protein on blood cells: potential functions in cell physiology and pathophysiology of transmissible spongiform encephalopathy diseases. *Transfus Med Rev, 15*(4), 268-281.

Wang, K. K., Zoltewicz, J. S., Chiu, A., Zhang, Z., & Rubenstein, R. (2012). Release of Full-Length PrP(C) from Cultured Neurons Following Neurotoxic Challenges. *Front Neurol, 3*, 147.

Warren, K. M., Reeves, T. M., & Phillips, L. L. (2012). MT5-MMP, ADAM-10, and N-cadherin act in concert to facilitate synapse reorganization after traumatic brain injury. *J Neurotrauma, 29*(10), 1922-1940.

Watt, N. T., Taylor, D. R., Kerrigan, T. L., Griffiths, H. H., Rushworth, J. V., Whitehouse, I. J., & Hooper, N. M. (2012). Prion protein facilitates uptake of zinc into neuronal cells. *Nat Commun, 3*, 1134.

Weinberger, S. (2011). Bombs' hidden impact: the brain war. *Nature, 477*(7365), 390-393.

Weise, J., Crome, O., Sandau, R., Schulz-Schaeffer, W., Bahr, M., & Zerr, I. (2004). Upregulation of cellular prion protein (PrPc) after focal cerebral ischemia and influence of lesion severity. *Neurosci Lett, 372*(1-2), 146-150.

Wopfner, F., Weidenhofer, G., Schneider, R., von Brunn, A., Gilch, S., Schwarz, T. F., . . . Schatzl, H. M. (1999). Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *J Mol Biol, 289*(5), 1163-1178.

Wroe, S. J., Pal, S., Siddique, D., Hyare, H., Macfarlane, R., Joiner, S., . . . Collinge, J. (2006). Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. *Lancet, 368*(9552), 2061-2067.

Yokobori, S., Hosein, K., Burks, S., Sharma, I., Gajavelli, S., & Bullock, R. (2013). Biomarkers for the clinical differential diagnosis in traumatic brain injury--a systematic review. *CNS Neurosci Ther, 19*(8), 556-565.

Yuan, A. D., Rao, M. V., Veeranna, & Nixon, R. A. (2012). Neurofilaments at a glance. *J Cell Sci, 125*(14), 3257-3263.

Zahn, R. (2003). The octapeptide repeats in mammalian prion protein constitute a pH-dependent folding and aggregation site. *J Mol Biol, 334*(3), 477-488.

Zetterberg, H., Hietala, M. A., Jonsson, M., Andreasen, N., Styrud, E., Karlsson, I., . . . Wallin, A. (2006). Neurochemical aftermath of amateur boxing. *Arch Neurol, 63*(9), 1277-1280.

Zhang, C. C., Steele, A. D., Lindquist, S., & Lodish, H. F. (2006). Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. *Proc Natl Acad Sci U S A, 103*(7), 2184-2189.

Zhao, J. B., Zhang, Y., Li, G. Z., Su, X. F., & Hang, C. H. (2011). Activation of JAK2/STAT pathway in cerebral cortex after experimental traumatic brain injury of rats. *Neurosci Lett, 498*(2), 147-152.

Zuegg, J., & Gready, J. E. (2000). Molecular dynamics simulation of human prion protein including both N-linked oligosaccharides and the GPI anchor. *Glycobiology, 10*(10), 959-974.